

**Press residues of berry seeds:
Ingredients and physiological importance following
human intervention studies**



since 1558

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LIST OF ABBREVIATIONS

15-keto-dihydro-PGF _{2α}	15-Keto-dihydro-Prostaglandin F _{2α}
8-iso-PGF _{2α}	8-Iso-Prostaglandin F _{2α}
8-oxodG	8-Oxo-2'-deoxyguanosine
AAs	Amino acids
AAR	Amino acid ratio
ABTS	(2,20-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt)
ADF	Acid detergent fibre
ADL	Acid detergent lignin
ANOVA	Analysis of variance
BIA	Bioelectrical impedance analysis
BHT	2,6-Di-tert-butyl- <i>p</i> -kresol (Butylated Hydroxytoluene)
BMBF	Federal Ministry of Education and Research
BMI	Body mass index
BQ	Biological quality
CEHC	Carboxyethyl-hydroxychromanol
CHD	Coronary heart disease
DACH	Germany, Austria, Switzerland
DAD	Diode array detector
DHBA	Dihydroxybenzoic acid
DM	Dry matter
DNA	Deoxyribonucleic acid
EAAI	Essential amino acid-index
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
FA	Fatty acid
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FDM	Fat in dry matter
GAE	Gallic acid equivalent
GC/FID	Gas chromatograph with flame ionisation detector

HDL	High density lipoprotein
HIS I	Human intervention study I (PR in yoghurt)
HIS II	Human intervention study II (PR in bread)
HPLC	High performance liquid chromatography
HT29	Human colon cancer cells
ICP-OES	Inductively coupled plasma optical emission spectrometry
IP ₆ (IP ₅ , IP ₄ , IP ₃)	Inositol hexaphosphate (and its intermediate phosphates)
LCFA	Long-chain fatty acids
LDL	Low density lipoprotein
MUFA	Monounsaturated fatty acids
NDF	Neutral detergent fibre
n.d.	Below detection limit
PI	Period I
PII	Period II
PII	Period III
PR	Press residue
PUFA	Polyunsaturated fatty acids
SCFA	Short-chain fatty acids
SD	Standard deviation
SFA	Saturated fatty acids
TEAC	Trolox equivalent antioxidant capacity
TOH _{Hydr}	Tocopherol- ω -hydroxylase (cytochrome P450)
UNU	United Nations University
UV	Ultraviolet detector
WHO	World Health Organization
α -TOH	α -Tocopherol
α -TTP	α -Tocopherol-transfer-protein
γ -TOH	γ -Tocopherol

INTRODUCTION AND OBJECTIVES

1 INTRODUCTION AND OBJECTIVES

1.1 INTRODUCING THE TEST SUBSTANCE AND AIM OF THE INVESTIGATIONS

Considering the fact that quantities of berry fruits are consumed worldwide and additionally studies indicate their potential for health, more scientific research must be targeted on this matter (SEERAM, 2008). Against that background, the present studying was conducted.

The black currant originates from the northern Asia and Europe and is nowadays cultivated in Europe, North America and New Zealand. Black currant is utilised for syrups, juices, jams, jellies, is added to berry fruit mixes and is used for the famous French Cassis liquor. Black currant but also other berry fruits are traditionally appreciated for diverse substances in promoting health in matters of antimicrobial activity and helicobacter pylori adhesion to the gastric mucosa of human, in matters of chemopreventive activity, atopic dermatitis, benefiting the immune response, or in affecting plasma lipids (YANG *et al.*, 1999; WU *et al.*, 1999; LANDBO & MEYER, 2001; XUE *et al.*, 2001; XING *et al.*, 2002; LENGSELD *et al.*, 2004; PUUPPONEN-PIMIÄ *et al.*, 2005; TAHVONEN *et al.*, 2005; TAKATA *et al.*, 2005; NOLI *et al.*, 2007). Anthocyanins of black currant reduced mediators of inflammation (KARLSEN *et al.*, 2007). The vitamin C concentration of the black currant berries is outstandingly high (189 mg/100 g according to PRODI[®] 5.4 software; NIELSEN *et al.*, 2003). But also phenolic structures, or antioxidant potential in general, minerals, fatty acids and fibres of the seeds contribute to the postulated health promoting properties of black currant (JOHANSSON *et al.*, 1997; MIKKONEN *et al.*, 2001; HALVORSEN *et al.*, 2002; SLIMSTAD & SOLHEIM, 2002; KÄHKÖNEN *et al.*, 2003; LU & FU, 2003; NIELSEN *et al.*, 2003; VEČEŘA *et al.*, 2003; VILJANEN *et al.*, 2004; WU *et al.*, 2004; MCDUGALL *et al.*, 2005; NARWIRSKA & KNAŚNIEWSKA, 2005). The leaves of black currant are declared as a remedy in the German Pharmacopoeia due to substances like tanning agents and rutin (GARBACKI *et al.*, 2005). Nevertheless, a generally low intake of fruits, vegetables and berries is associated to the occurrence of cardiovascular diseases (RISSANEN *et al.*, 2003). To exploit the full potential of the berry fruit, also the seeds should be utilised. They are a most widely unobserved matrix and analyses so far not exceed oils and the containing tocopherols. Concerning health aspects, these analyses revealed good quality fatty acid patterns or tocopherol and tocotrienol concentrations (JOHANSSON *et al.*, 1997; IVANOFF & AITZETMÜLLER, 1998; JOHANSSON *et al.*, 2000; OOMAH *et al.*, 2000; GOFFMAN & GALLETTI, 2001; KALLIO *et al.*, 2002a; KALLIO *et al.*, 2002b; RUIZ DEL CASTILLO

et al., 2002; BUSHMAN *et al.*, 2004; KALLIO *et al.*, 2005; PARRY *et al.*, 2005; STRÁNSKÝ *et al.*, 2005). Other ingredients of the seeds were not observed to a larger extent so far. Black currant seed oil is gained from seeds by default. During this process a press residue (PR) remains, which is considered as a waste product at the present though it is still rich in oils. To find out more about the ingredients and potential of the seed PR of black currant, but also from the PR of other common berries, this work was conducted. PR analysed were bilberry (*Vaccinium myrtillus* L.), cranberry (*Vaccinium oxycoccus* L.), rose hip (*Rosa canina* L.), strawberry (*Fragaria x ananassa* L.), elder (*Sambucus nigra* L.) and two different batches of black currant (*Ribes nigrum* L.) (FIGURE 1).

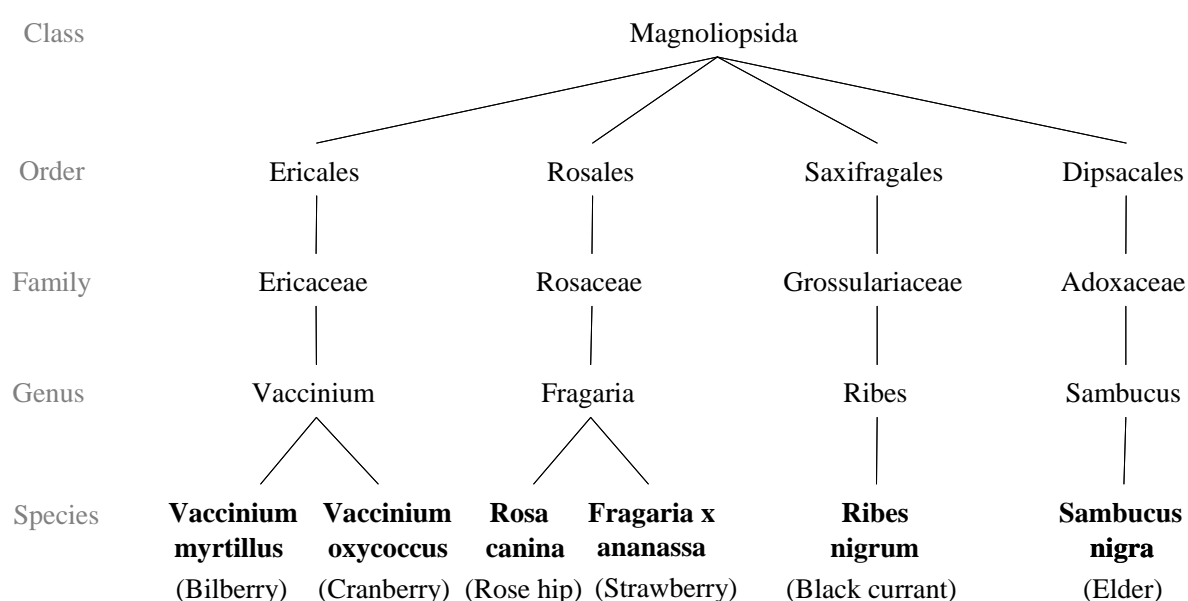


FIGURE 1 Taxonomic classification of tested species.

After determining the main and presumed minor substances of the PR, the PR with the most promising health-promotion was assessed in order to be tested for its applicability into foods. It was further assessed, which substances might cause measurable effects during a human intervention and which of these resulting effects might be meaningful. As a second selection criterion it was raised which species of seeds was obtainable on the market in abundance. In consequence of these deliberations, the decision was made on black currant PR. After that, it was estimated, whether the designed food has potential to serve as a functional food; according to the EU Regulation 258/97, functional foods need to be added as part of the normal diet with verifiable health promoting effects at ordinary consumption portions.

1.2 SEED PRESS RESIDUES AND PHYSIOLOGICAL RELEVANCE OF MAIN CHARACTERISTICS

The purchased seeds were pressed. The remainder formed as pellets underwent processing such as grinding and sieving in order to reach an acceptable mouth feeling. After that, the procedure allowed a consumable product having a particle size $<500\ \mu\text{m}$ (FIGURE 2).



a) Black currant seed press residue

b) Whole grain bread including 8% black currant seed press residue

FIGURE 2 Black currant seed press residue pure (a) and baked into whole grain bread (b).

PR were screened with different methods to get an overall evaluation of the matrix (MANUSCRIPT I). Bilberry, cranberry, rose hip, strawberry, elder and black currant were tested for their concentrations of oil and fatty acid pattern, their concentration of tocopherols and tocotrienols, vitamin C and total fibre, gallic acid equivalent and Trolox equivalent antioxidant capacity (TEAC). In black currant further more dry matter, ashes, protein concentration and quality, concentration of the fibre fractions NDF and ADF, inositol phosphates, anthocyanins, minerals, plant sterols, carotenoids and energy content were analysed.

➤ TOCOPHEROLS

Daily losses of vitamin E are estimated to be about 15 mg (BRUNO *et al.*, 2006 a). This loss defines the dose recommended to be renewed with the daily diet to remain an adequate serum tocopherol concentration. However, a study by MITMESSER *et al.* (2000) showed, more than one third of the examined adults had low serum vitamin E concentrations of $<20\ \mu\text{mol/l}$.

Tocopherols and tocotrienols are vitamins comprising different structures (FIGURE 3). The vitamin activity of the isomers decreases with the reduced number of methyl groups in the

chromanol ring and further depends on their position on the ring and saturation of the lipophilic side chain (PONGRACZ *et al.*, 1995). As described above berry seeds are a natural source of tocopherols and tocotrienols. Their bioavailability differs according to their surrounding food matrices (REBOUL *et al.*, 2006). Being essential, vitamin E needs to be exogenously added to the body. After absorption in the gut, tocopherol reaches the liver where the α -tocopherol transfer protein exists. This protein preferentially binds α -tocopherol and incorporates it into VLDL (KAEMPF-ROTZOLL *et al.*, 2003). This is the reason for α -tocopherol being the main tocopherol isomer in the blood.

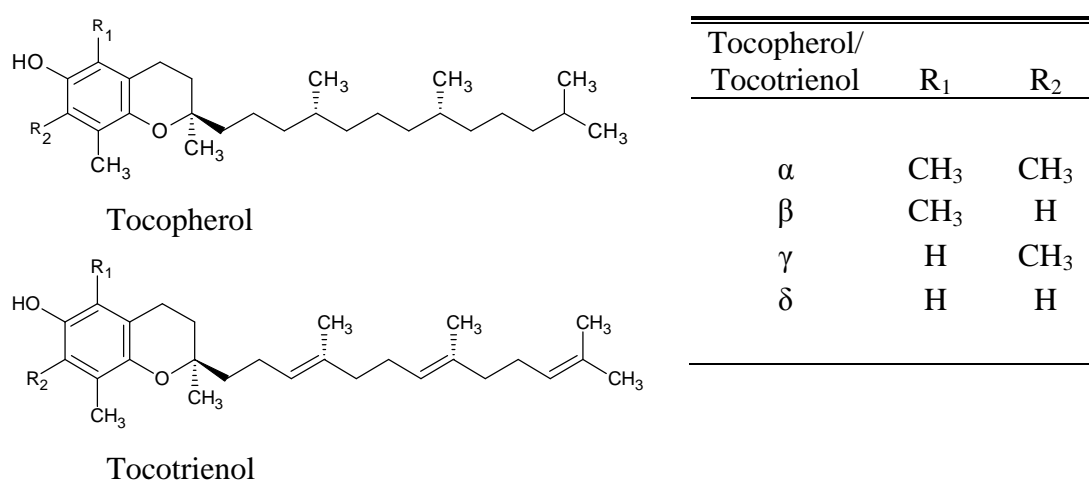


FIGURE 3 Chemical structure of tocopherols and tocotrienols.

α -Tocopherol is regarded as the isomer with the greatest vitamin activity. Tocotrienols have a lower vitamin activity than their corresponding tocopherols. Tocopherols are thermally stable. Vitamin E has relevance in the body for different reasons. α -Tocopherol has antioxidant properties in lipoproteins and reduces the urinary concentration of markers of lipid peroxidation in rats (ROOB *et al.*, 2000; SÖDERGREN *et al.*, 2000; MILNE *et al.*, 2005). Further, in low concentrations it is neuroprotective by suppressing certain signal transductions (KHANNA *et al.*, 2003). γ -Tocopherol has an electrophilic character and is able to detoxify nitrogen dioxide (COONEY *et al.*, 1995; SACHDEVA *et al.*, 2005). Comparing α - and γ -tocopherol in different cell systems showed, only γ -tocopherol but not α -tocopherol had chemopreventive effects such as reducing cell proliferation or inducing apoptosis (CAMPBELL *et al.*, 2006). In prostate cancer the serum concentration of both, α - and γ -tocopherol is decreased (WEINSTEIN *et al.*, 2005). An inverse association between serum α -tocopherol concentration and breast cancer risk is described (KIM *et al.* 2001). Further, it was shown that

women being vitamin E-supplemented have a lowered coronary heart disease risk (STAMPFER *et al.*, 1993). STOCKER *et al.* (1999) concluded that if tocopherols prevent atherosclerotic events, then not by working as a classic antioxidant, but for example by immunological regulation. In patients suffering from coronary heart diseases, the serum γ -tocopherol concentration is decreased (KONTUSH *et al.*, 1999). Nonetheless, in plaques the oxidised lipoproteins and α -tocopherol coexist and in arteriosclerotic events vitamin E is not depleted, neither does its presence prevent lipid oxidation of the arterial walls, which might explain why vitamin E applied solely fails to demonstrate antiatherogenic effects in many studies (NIU *et al.*, 1999; UPSTON *et al.*, 2002). WAGNER *et al.* (2004) summarises that evidence for tocopherols in regard to coronary heart disease and cancer risk was only possible when involving the γ -tocopherol next to α -tocopherol. INGLES *et al.* (1998) reasoned that a high α -/ γ -tocopherol ratio of plasma could be a better indicator for a reduced cancer risk than α -tocopherol alone. In rats, γ -tocopherol and its metabolite γ -carboxyethyl-hydroxychromanol (CEHC) reduced proinflammatory eicosanoids and damage caused by inflammation (JIANG & AMES, 2003). Finally, γ -tocopherol has potential not fully exploited, but likely not by working with its radical scavenging ability, but by growth reduction and promoting apoptosis in cancer cell lines, or by removing peroxynitrite-derived nitrating species (CHRISTEN *et al.*, 1997; CAMPBELL *et al.*, 2006).

Manifested vitamin E-deficiency is rare and only present in individuals suffering from fat malabsorption or enzyme defects, but marginal deficiency may exist in considerable extend among the population (MEYDANI, 1995). A damage causing from augmented intake is not to expect *per se*, because intake above demand would enter intensified excretion *via* bile or metabolites *via* kidney (SCHULTZ *et al.*, 1995; SCHUELKE *et al.*, 2000). According to MILLER *et al.* (2005), all-cause mortality may increase at high doses of vitamin E supplements. However, vitamin E should accompany substrates sensitive towards oxidants, because for example at long-term intake of fish oil capsules, the antioxidant status of the body gets diminished and plasma lipid peroxides increased (MEYDANI *et al.*, 1991). The diversity in studies dealing with tocopherols and tocotrienols in regard to both antioxidant and non-antioxidant attributes demonstrates the diversity of the far-ranging effects of vitamin E, its potential but also the still existing lack of clarity of its mode of action. Concluding, effects and mode of action of vitamin E still are of big interest.

➤ *FIBRE*

The class of fibres includes many different chemical structures. What they all have in common is their non-digestibility by human enzymes of the gastrointestinal tract (CUMMINGS *et al.*, 2001). According to the dietary guidelines of the DACH references, a daily intake of 30 g fibre should be aimed (GERMAN NUTRITION SOCIETY, 2000). However, according to the National Nutrition Survey II a mean of only 23 g/d (women) and 25 g/d (men) are achieved (MAX RUBNER-INSTITUT, 2008).

Fibres are classified into soluble and insoluble fibres. The water-holding capacity of stool is closely related to the amount of the respective fibre fractions in the stool, but also of their granulometry (ESPOSITO *et al.*, 2005). Due to the flexible structure of the fibre surface, they are able to bind and enclose water molecules including the dissolved substances (CHAPLIN, 2003). In this way, fibre may further partition and possibly delay the absorption of hydrophobic solvents containing tocopherols or toxins resulting from foods or metabolism (HARRIS *et al.*, 1998). The binding abilities of fibres are varying. Dietary fibres from rye reduced transit time in pigs, which reduces the exposure of enterocytes towards toxins (GLITSØ *et al.*, 1998).

Fibres also bind and metabolise cholesterol *via* gut microbiota more or less intense (SEMBRIES *et al.*, 2004). Consequently, cholesterol excretion and transformation (e.g. to neutral sterols) can increase by fibre consumption. Again, the quality of fibre determines the extent of it (DONGOWSKI *et al.*, 2002). Increased steroid excretion is supposed to be beneficial because it may result in a lowering of serum cholesterol concentration (CHAU *et al.*, 2004). Some fibres increase the short-chain fatty acid formation in the gastrointestinal tract which is considered to be favourably due to promoting expression of protective enzymes, for example (VON ENGELHARDT *et al.*, 1989; ABRAHAMSE *et al.*, 1999; SANDERSON, 2004; BACH KNUDSEN *et al.*, 2005; POOL-ZOBEL *et al.*, 2005; WONG *et al.*, 2006).

It was shown that a diet low in fibre and high in fat and meat increases the formation of hydroxyl radicals in stool, which may increase the risk of colorectal cancer (ERHARDT *et al.*, 1997). Fibres can inhibit the growth of tumour cells *in vitro* (BEYER-SEHLMAYER *et al.*, 2003). Further, fibre rich cereals can have a high antioxidant capacity, which might be attributed to the accompanying fibre-bound phenolic compounds (ESPOSITO *et al.*, 2005). After all, mechanisms of action by fibre in regard to reduce the colon cancer risk are speculative (LIM *et al.*, 2005).

➤ *PHYTIC ACID*

Phytic acid (inositol hexaphosphate or IP₆) and its lower phosphorylated inositols IP₁-IP₅ are present in grains, legumes, oil seeds and nuts (GRAF *et al.*, 1987). In plants, these molecules serve to store phosphorus, cations and high energy phosphoryl groups or to regulate cellular functions (IP₁-IP₄) (SHAMSUDDIN, 2002). The phytic acid concentration in wheat brown bread is 0.7-1.6 mmol/100 g, in white bread it is <0.5µmol/100 g (BOHN *et al.*, 2004). It is demonstrated in the rat stomach and rat upper intestine that phytic acid is quickly absorbed (SHAMSUDDIN, 2002). Phytic acid and its phosphates can be recovered in virtually all mammalian cells. It is shown for the human organism that phytic acid reduces mineral absorption by forming insoluble and thus not absorbable complexes in the intestine at a pH >6 (BOHN *et al.*, 2004). It is reported, that after daily and long-term consumption of fibre-rich wheat bread naturally containing phytic acid, the iron status decreased in young women (BACH KRISTENSEN *et al.*, 2005). Phytic acid inhibited induced carcinogenesis in rats and increased the activity of natural killer cells (SHAMSUDDIN, 2002; ZHANG *et al.*, 2005). This can be attributed to the potentially antioxidant properties of phytic acid, though it is uncertain, if physiological intakes of phytic acid are able to improve the antioxidant status of an organism (GRAF *et al.*, 1987; RIMBACH & PALLAUF, 1998; MINIHANE & RIMBACH, 2002).

➤ *OXIDATIVE STRESS*

Reactive oxygen species are highly reactive oxygen metabolites physiologically formed by the mitochondrial electron transport in the respiratory chain and various hydroxylation and oxygenation reactions of the body with various outcomes (McCORD & FRIDOVICH, 1970; MOLDOVAN & MOLDOVAN, 2004; MULLER *et al.*, 2007). Their formation can be augmented by events of disease and extensive physical activity for example (CASTRO & FREEMAN, 2001). Their reactivity by instable electron configuration makes them gain electrons from other molecules, *e.g.* lipids, proteins or DNA (VALKO *et al.*, 2004; PASSOS & VON ZGLINICKI, 2006). Most of them have a short half-live span what makes them hard to detect (MOLDOVAN & MOLDOVAN, 2004). This circumstance requires special test systems to discover their effects in the body. The body posses many defence systems towards reactive oxygen species, but an imbalance of oxidants and antioxidants can stress the body, which is then called oxidative stress. Not at last, an elected nutrition can affect the defence systems of the body. Certain food components have protective effects, *e.g.* vitamins like vitamin C and E, carotenoids, polyphenols or selenium (VALKO *et al.*, 2004). Other food components promote oxidative

stress reactions, *e.g.* PUFA and iron intake or toxins (JENKINSON *et al.*, 1999; VALKO *et al.*, 2006).

The effects of oxidative stress can be measured by means of biomarkers such as urinary 8-oxo-2'-deoxyguanosine (8-oxodG, oxidative DNA-damage repair product), urinary F₂-isoprostanes and prostaglandin F_{2α} metabolites (products of lipid peroxidation), genotoxicity of faecal water (comet assay), or the antioxidant capacity in urine or serum (hydroxylation products of salicylic acid, TEAC assay).

1.3 HUMAN INTERVENTION STUDIES

By means of two human intervention studies, the potential health benefits and physiological properties of black currant PR were evaluated. At the first human intervention study (HIS I) with 18 female smokers, the black currant PR was tested in regard to dose effects (FIGURE 4).

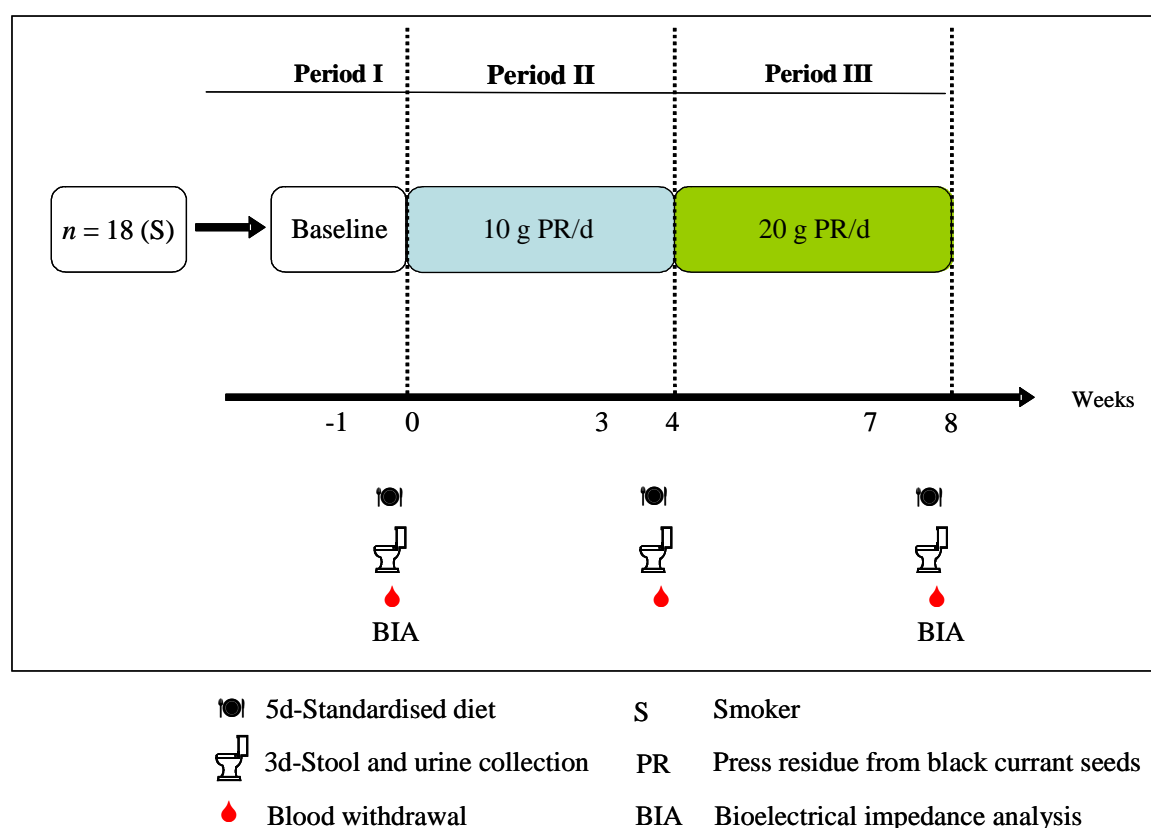


FIGURE 4 Design of a human intervention study on the consumption of different doses of black currant seed press residue. PR was consumed in yoghurt, which was applied throughout the entire study (150 g/d, periods I-III).

10 g PR/d and 20 g PR/d were applied in pure yoghurt for four weeks each compared to a baseline period. The samples stool, urine, and blood were collected at standardised conditions during the last five days of each period. At these five days, a standardised diet was administered which was weighed and prepaced and daily comprised a warm meal, fruits and vegetables, bread, bread-spreads and beverages (TABLE A-1). All uneaten portions were returned for quantification of the actual food consumption.

TABLE 1

Concentrations of different parameters during a 5-day standardised diet with 3-day stool collection following consumption of black currant seed press residue (PR) in yoghurt (means \pm SD; $n = 18$).

	Unit	Baseline	10 g PR/d	20 g PR/d
Fibre intake	[g/d]	27.7 \pm 3.9 ^c	32.4 \pm 5.1 ^b	35.4 \pm 6.8 ^a
Fibre excretion	[g/d]	8.88 \pm 3.07	9.63 \pm 4.25	9.63 \pm 3.61
Tocopherol intake	[μ mol/d]	16.4 \pm 1.7 ^c	19.5 \pm 2.4 ^b	23.0 \pm 3.3 ^a
Total tocopherol serum	[μ mol/L]	32.1 \pm 6.6	31.1 \pm 6.4	31.5 \pm 8.0
Total tocopherol stool	[μ mol/d]	17.8 \pm 7.6	19.1 \pm 9.9	20.1 \pm 10.4
Neutral sterols (sum)	[mg/g]	7.39 \pm 5.04 ^a	6.00 \pm 4.42 ^b	5.16 \pm 3.24 ^b
8-oxodG urine	[nmol/kg/24h]	1.63 \pm 2.50	1.35 \pm 2.11	1.36 \pm 1.65
Comet assay *	[Tail intensity]	12.7 \pm 5.7 ^c	15.9 \pm 8.81 ^b	22.5 \pm 14.9 ^a
Cytotoxicity stool *	[% cell viability]	97.7 \pm 2.1 ^a	97.4 \pm 3.5 ^a	95.1 \pm 4.6 ^b
Urinary 8-iso-PGF _{2α}	[mg/d]	not analysed	2.67 \pm 1.06	3.53 \pm 1.83
Urinary 15-keto-dihydro-PGF _{2α}	[mg/d]	not analysed	0.71 \pm 0.23	0.88 \pm 0.51
Serum ferritin	[μ g/L]	45.8 \pm 33.2 ^a	38.4 \pm 25.2 ^b	36.8 \pm 26.2 ^b
Vitamin C urine	[mmol/d]	350 \pm 239	317 \pm 223	307 \pm 219
TEAC urine	[mmol/L]	4.73 \pm 3.21	4.35 \pm 2.58	4.90 \pm 2.57

^{a,b,c}Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

Statistical evaluation was carried out using analysis of variance for repeated measures (SPSS 14.0 software package, SPSS Inc., Chicago, IL, USA).

8-oxodG: 8-oxo-2'-deoxyguanosine; DHBA: dihydroxybenzoic acid; 8-iso-PGF_{2 α} : 8-iso-prostaglandin F_{2 α} ; 15-keto-dihydro-PGF_{2 α} : 15-keto-dihydro-prostaglandin F_{2 α} ; TEAC: Trolox equivalent antioxidant capacity

* Tested using HT29 clone cells treated with 10% faecal water.

Compared to baseline, both intervention dosages caused a significant increase in fibre and tocopherol intake, genotoxicity of faecal water and a significant decrease in neutral sterol excretion (TABLE 1). No general differences could be verified between the both dosages besides significantly increased fibre and tocopherol intake and significantly increased genotoxicity and cytotoxicity of faecal water for the 20 g/d dosage compared to the 10 g/d dosage.

To test the PR in a processed food, a second human intervention study (HIS II) was conducted (FIGURE 5). PR was incorporated into bread because this food provided a neutral and appropriate matrix for a convenient PR intake compared to other foods like *e.g.* pizzas, sausages, or ready-made meals (FIGURE 2).

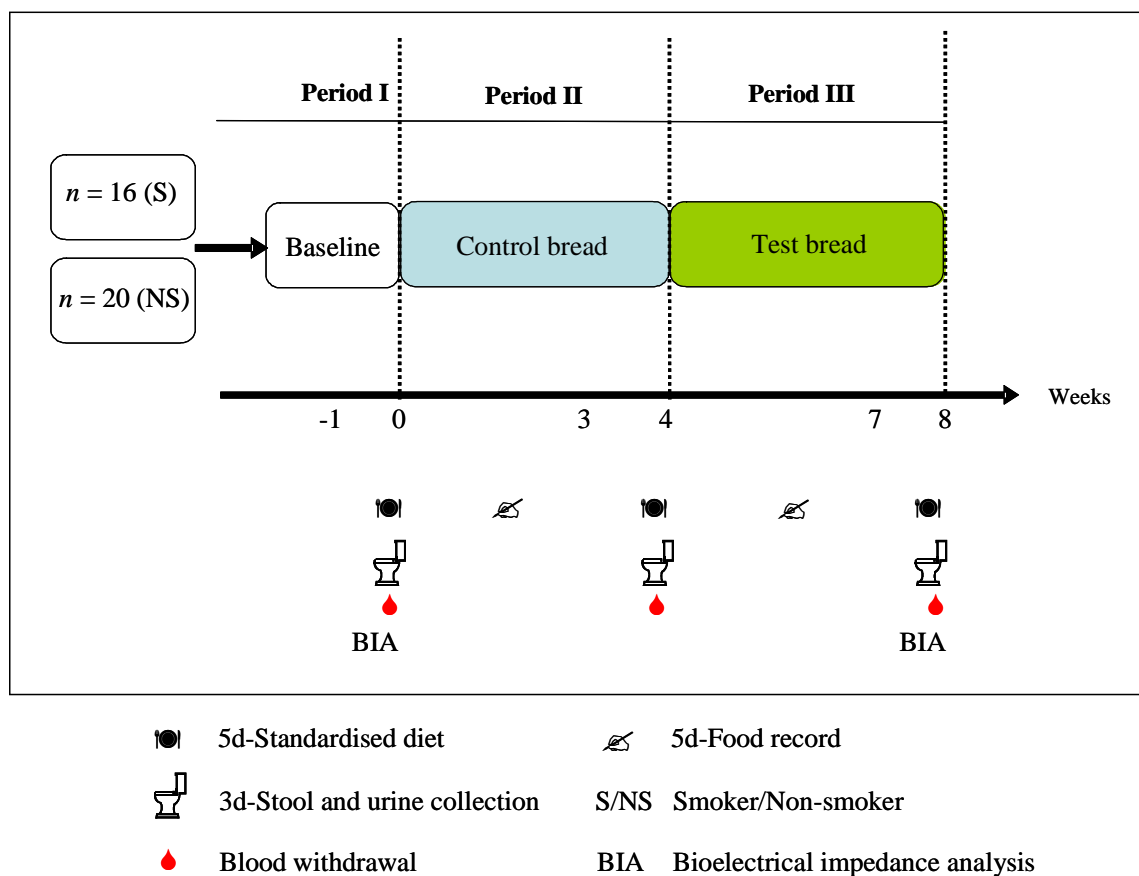


FIGURE 5 Design of a human intervention study on the consumption of black currant seed press residue baked in whole grain bread (test bread) compared to a control bread and baseline data.

In accordance with the results of the first study, the second study was carried out using the dosage of 20 g PR/d which resulted in a bread intake of 250 g/d. The study design and duration was identical to HIS I.

Outcomes of the berry seed PR analyses of the different species with special focus on black currant seed PR and the physiological effect after consumption of black currant seed PR particularly consumed in bread are given in the following MANUSCRIPTS I-III and the final discussion.

1.4 OBJECTIVES

From the above described approaches, the following both main objectives can be summarised:

1. Characterisation of the substances of content in different berry seed PR including the specification of main ingredients.

Regarding the potential of the PR to be part of a functional food:

2. Testing on physiological effects and health-benefits after oral administration of berry seed PR at different dosages (HIS I) and its testing after application of berry seed PR when processed during food fabrication (HIS II).

OVERVIEW OF MANUSCRIPTS

2 OVERVIEW OF MANUSCRIPTS

MANUSCRIPT I (PUBLISHED)

Berry seed press residues and their valuable ingredients with special regard to black currant seed press residues

Dorit Helbig, Volker Böhm, Andreas Wagner, Rainer Schubert, Gerhard Jahreis
Food Chemistry 111:1043-1049 (2008)

CONDENSED ABSTRACT

The paper deals with the characterisation and general composition of press residue substances of different berry seeds. Regarding a physiological health impact, fibres and tocopherols are the most promising substance groups of the press residues. Special focus is provided to black currant seed press residue.

INFORMATION ABOUT OWN CONTRIBUTION

DH was responsible for the supervision of the human study, sample handling and for conducting the analyses as well as writing of the manuscript; VB supported analyses of total phenols (gallic acid equivalent), antioxidant capacity (TEAC) and vitamin C; AW supported tocopherol analyses; RS was responsible for obtaining funding and supported statistical evaluation; DH and GJ were responsible for data interpretation.

MANUSCRIPT II (ACCEPTED)

Black currant seed press residue increases tocopherol concentrations in serum and stool whilst biomarkers in stool and urine indicate increased oxidative stress in humans

Dorit Helbig, Andreas Wagner, Michael Gleis, Samar Basu, Rainer Schubert,
Gerhard Jahreis

British Journal of Nutrition Accepted 5 December 2008

(doi:10.1017/S0007114509220812)

CONDENSED ABSTRACT

Black currant seed press residues were baked into bread and tested in the course of a human intervention study. The press residue contained antioxidant substances (e.g. tocopherols), thus effects in this regard were analysed and discussed. Biomarkers of exposure, namely the tocopherol concentration in serum, increased. At the same time, the concentrations of biomarkers of effect associated with oxidative damage (tail intensity in HT-29 by faecal water, hydroxylation products of salicylic acid in faeces) increased, too.

INFORMATION ABOUT OWN CONTRIBUTION

DH was responsible for the supervision of the human study, sample handling and for conducting the analyses as well as writing of the manuscript; AW supported tocopherol, 8-oxodG and DHBA analyses; MG supported the analyses of cell viability and the performance of the comet assay; SB was responsible for the prostane analyses; RS was responsible for obtaining funding and supported statistical evaluation; DH and GJ were responsible for data interpretation; all authors were responsible for critical revision of the manuscript.

MANUSCRIPT III (SUBMITTED)

Tocopherol isomer pattern in serum and stool of human following consumption of black currant seed press residue administered in whole grain bread

Dorit Helbig, Andreas Wagner, Rainer Schubert, Gerhard Jahreis

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CONDENSED ABSTRACT

Black currant seed press residue baked into bread yields in tocopherol and fibre rich bread. This bread was tested on physiological effects in a human intervention study. As special feature, influences of the serum γ -tocopherol concentration and the faecal fibre were focused in particular.

INFORMATION ABOUT OWN CONTRIBUTION

DH was responsible for the supervision of the human study, sample handling and for conducting the analyses as well as writing of the manuscript; AW supported tocopherol analyses; RS was responsible for obtaining funding and supported statistical evaluation; DH and GJ were responsible for data interpretation; all authors were responsible for the critical revision of the manuscript.

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Analytical Methods

Berry seed press residues and their valuable ingredients with special regard to black currant seed press residues

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ABSTRACT

Berry seeds are distinguished by longevity though clear scientific appraisals cannot be made. Besides a hard seed coat other protecting substances are presumed in the seeds. Commonly the seeds are utilized as a source of oils. After pressing, there is a residue left that is still rich in bioactive ingredients. This paper gives an overview of the health-beneficial ingredients remaining in the residue of various berry seeds (bilberry, cranberry, rose hip, strawberry, elder, and black currant) with special focus on black currant. The fatty acid distribution and the content of fat, tocopherols and tocotrienols, phytosterols, carotenoids, vitamin C, fibre, protein, amino acids, dry matter, ashes, minerals, total phenols (gallic acid equivalent) and antioxidant capacity (TEAC) were determined. The investigation of berry seed press residues revealed that the total phenols and tocopherols were quantitatively the most important features of this material but there were significant differences between batches and cultures.

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1. Introduction

In the production of berry-based juices, jellies and jams, most processors consider the seeds of the berries to be removed from the berry pulp during processing to improve the end product. The berry seed materials removed as waste by-products may contain health-beneficial compounds. As a consequence, commercial uses should be found. Since little, hard seeds are usually excreted undigested, assumed health-promoting substances inside the seeds cannot develop their potential. Therefore, the processed seeds might be beneficial. Investigations were undertaken to determine the chemical composition of berry seeds and to characterize some of the potentially health-beneficial substances. Previous research focused mainly on the seed oil fatty acid (FA) composition (Johansson, Laine, Linna, & Kallio, 2000; Ruiz del Castillo, Dobson, Brennan, & Gordon, 2002), triacylglycerols, and glycerophospholipids (Kallio, Yang, Peippo, Tahvonen, & Pan, 2002b) or tocopherols and tocotrienols (Kallio et al., 2002b; Bushman et al., 2004). Oil seeds have been examined for FAs to promote health and to prevent disease, e.g., atopic dermatitis (Noli et al., 2007), gastric ulcer (Xing et al., 2002), immune response (Wu et al., 1999) or effects on plasma lipids (Tahvonen, Schwab, Linderborg, Mykkänen, & Kallio,

2005). Furthermore, research was conducted on the fibre (Nawirska & Kwaśniewska, 2005), phytosterols (Yang, Koponen, Tahvonen, & Kallio, 2003), polyphenols (Lu & Foo, 2003) and the antioxidant potential of the seeds or the seed oils (Bushman et al., 2004; Parry et al., 2005). The protective effect of black currant seeds towards *helicobacter pylori* adhesion to the gastric mucosa of human was investigated by Lengsfeld, Deters, Faller, and Hensel (2004). Black currant seeds are recommended as edible inclusion in breakfast cereals (Tahvonen et al., 1998). High nut and seed intakes (>6.2 g/d, e.g., sunflower, pumpkin) were associated with a decreased risk of colon cancer in women, but this effect could not be demonstrated in men (Jenab et al., 2004). The above mentioned literature does not specifically indicate whether health-promoting constituents are also present in the berry seeds. The purpose of the present investigation was to quantify and to characterize the berry press residue overall attributes.

2. Material and methods

2.1. Treatment of the berry seeds

Seeds of bilberry (*Vaccinium myrtillus* L.), cranberry (*Vaccinium oxycoccus* L.), rose hip (*Rosa canina* L.), strawberry (*Fragaria x ananassa* L.), elder (*Sambucus nigra* L.), and black currant (batch I and II) (*Ribes nigrum* L.) were obtained from a wholesale trader who

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provided seeds in large amounts. This trader buys seeds throughout countries of the European Community. As a consequence, seeds of different growing areas can be pooled and heritage may vary from year to year. Detailed growing conditions of berry plants cannot be verified. Berry seed samples were pressed at approximate 60 °C with varying temperatures in different segments of the press procedure. Frictional heating was an appreciable contributor to the heat development. Oil was removed for purposes not mentioned in this paper. The seed press residue (PR) was ground and sieved to a particle size <500 µm (IGV Potsdam). Thus, hard coat fractions were separated and PRs could be utilized as a fortification in edible products. PRs were stored at –20 °C until analyses.

2.2. Fat content and FA distribution

An aliquot of the sample was boiled with 4 N HCl for 1 h, filtrated, washed with HPLC-grade water and extracted twice with petroleum ether using a Soxhtherm®. The total fat content was gravimetrically determined. For determination of total lipids, the extracted fat was methylated using sodium methoxide in the presence of hexane, then mixed with sodium hydrosulphate, and centrifuged. The supernatant fraction was analyzed by GC/FID using H₂ as carrier gas (Kraft, Collomb, Möckel, Sieber, & Jahreis, 2003). The used column is qualified to separate fatty acid methyl esters (FAME) between C₄ and C₂₂. Results are given as percent of total FAME. Calibration standard for FAME (no. 18919-1AMP) was purchased at Supelco, Taufkirchen, Germany.

2.3. Tocopherols and tocotrienols

Vitamin C was added to an aliquot of the sample followed by a hydrolysis with 2 M ethanolic KOH (80%) at 80 °C for 30 min. The tocopherols/tocotrienols were extracted with *n*-hexane/2,6-di-*tert*-butyl-*p*-kresol and determined using HPLC/fluorescence (Kuhnt, Wagner, Kraft, Basu, & Jahreis, 2006). A standardized milk powder (BCR-421, Promochem, Wesel, Germany) with a known content of tocopherol was analyzed in parallel with each sample batch. Standards for tocopherol calibration (Isomer kit no. 613424) were purchased at Calbiochem, Darmstadt, Germany and for tocotrienol (Isomer kit no. 8524) at Merck, Darmstadt, Germany.

2.4. Phytosterols

An aliquot of the sample with an internal standard (5 α -Cholesterol, no. C8003, Sigma-Aldrich, Taufkirchen, Germany) was hydrolysed at 70 °C for 1 h in 1 M ethanolic NaOH (90%) followed by three extractions with cyclohexane. Combined extracts were concentrated under a stream of nitrogen and redissolved in *n*-decane for measurement of phytosterols using GC/FID under isocratic conditions with H₂ as carrier gas (Keller, Helbig, Härtl, & Jahreis, 2007). Sitosterol (no. C1270) and campesterol (no. C5157) for calibration were purchased at Sigma-Aldrich, Taufkirchen, Germany.

2.5. Carotenoids

An aliquot of the sample and internal standard (echinenone, CaroteNature, Lupsingen, Switzerland) was extracted after addition of MgO with tetrahydrofuran/methanol with 0.1% butylated hydroxytoluene (1 + 1 v/v) according to Seybold, Fröhlich, Bitsch, Otto, and Böhm (2004). The extract was vacuum-dried at 30 °C and redissolved in tetrahydrofuran/methanol with 0.1% butylated hydroxytoluene (1 + 1, v/v). The content of carotenoids was measured using gradient procedure at HPLC/DAD. Standards for all carotenoids investigated were purchased at CaroteNature, Lupsingen, Switzerland.

2.6. Antioxidant capacity (TEAC), total phenols (gallic acid equivalent)

Antioxidant capacity was assessed by the TEAC-assay (Trolox equivalent antioxidant capacity) according to Schlesier, Harwat, Böhm, and Bitsch (2002). After an aliquot of the sample was extracted with *n*-hexane (TEAC II) or distilled water (TEAC III), ABTS (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt) was added which was prior to the test oxidised to a radical with MnO₂ (TEAC II) or K₂O₈S₈ (TEAC III). The sample was photometrically measured at $\lambda = 734$ nm using Trolox® (Sigma-Aldrich, Taufkirchen, Germany) standard solutions for quantification.

Total phenols were determined by the Folin-Ciocalteu method (Schlesier et al., 2002). An aliquot of the sample was dissolved in HPLC-grade water. To the supernatant fraction Folin-Ciocalteu reagent (Fluka, Buchs, Switzerland, 1:10 diluted) and Na₂CO₃ · 10H₂O was added and mixed. After 2 h reaction time, absorbance at $\lambda = 750$ nm was determined. Results are presented as gallic acid equivalents (GAE) with gallic acid monohydrate as standard (Riedel-de Haën, Seelze, Germany).

2.7. Vitamin C

Dehydroascorbic acid, the oxidised form of vitamin C, was determined by photometric analysis according to Schlesier et al. (2002). In brief, a sample aliquot diluted in phosphoric acid was mixed with trichloroacetic acid and centrifuged. Supernatant fraction was heated with dinitrophenylhydrazine reagent at 60 °C for 1 h. Samples were cooled in an ice bath and mixed with sulfuric acid before measurement. Results were calculated using a standard curve with different concentrations of ascorbic acid (Fluka, Buchs, Switzerland).

2.8. Fibre

Total fibre was determined enzymatically with BIOQUANT® Total Dietary Fibre (Merck, Darmstadt, Germany) employing α -amylase, protease, and amyloglucosidase (AOAC International, 1995). Detergent analysis was carried out according to the Van Soest method estimating NDF (neutral detergent fibre), ADF (acid detergent fibre) and ADL (lignin) (Van Soest, Robinson, & Lewis 1991). In brief, aliquots of the sample were boiled with the NDF and ADF solutions, respectively, and subsequently washed with distilled water. The respective fibre fractions were determined gravimetrically after drying and incinerating. For ADL, another ADF-fraction was hydrolyzed with 72% H₂SO₄, then washed with distilled water and acetone and dried. Ash content was determined and mass loss was recorded. Hemicelluloses were calculated as NDF-ADF, cellulose as ADF-ADL.

2.9. Protein and amino acids

For crude protein analysis the Kjeldahl method was applied ($N \times 6.25$).

For the determination of acid (Asp, Glu), neutral (Thr, Ser, Pro, Hyp, Ala, Gly, Val, Ile, Leu, Tyr, Phe) and alkaline (Lys, His, Arg) amino acids (AAs), a prior acid hydrolysis was used with 6 N HCl. Due to their instability, sulphur-containing AAs (Met, Cys) needed to be oxidised to methionine sulfon and cysteic acid using performic acid, before acid hydrolysis followed. Since the neutral AA tryptophan would be destroyed at those conditions, the sample was hydrolysed with 4 N LiOH at 110 °C for 20 h under anaerobic conditions (Landry, Delhaye, & Jones 1992). AAs were verified by ion exchange chromatography with ninhydrin post column derivatization (Amino Acid Analyzer LC 3000; Eppendorf/Biotronik). Calibration standards were purchased at Onken GmbH, Gründau, Germany.

To evaluate the protein quality, the amino acid ratio (AAR), the essential amino acid-index (EAAI) and the biological quality (BQ) were calculated as written below

- AAR = g of AA per 16 g N of test protein ÷ requirement [g] of AA per 16 g N.
(AAR is based on the AA requirements from the FAO/WHO/UNU, 1985).
- EAAI = geometric mean of the AAR of all essential AAs.
- BQ = $-15.71 + 1.0975 \times \text{EAAI}$ (approximated BQ).

EAAI and BQ are based on the pattern of reference protein from whole egg.

2.10. Dry matter, ash content, and elements

Dry matter was determined gravimetrically after drying at 105 °C for 24 h. Ash content was determined after sample conditioning at 525 °C for 4 h. For mineral determination 2.5 mL 25% HCl were added to the ash sample, incubated for 5 min and the flask was filled up with H₂O to a final volume of 25 mL. The sample was heated, filtrated and acidified with ultrapure HNO₃ to a final concentration of 2% of the sample. For measuring with inductively coupled plasma optical emission spectrometry (ICP-OES; Spectroflame, Spectro, Kleve, Germany), samples were 1:2 diluted with ultrapure water before injection. Analyses were conducted according to DIN 38406 (E22) except for Al, which was determined at $\lambda = 167.08$ nm. Dissenting from the DIN, calibration ranges for the elements were adjusted according to the expected concentrations. Calibration standards were purchased at Merck, Darmstadt, Germany (multielement standard Merck IV; As-, Mo-, Se-, and Ti-ICP standard) and Alfa Aesar, Karlsruhe, Germany (P-, S-, V-plasma standard).

3. Results and discussion

3.1. FAs

Seeds were pressed in order to be removed from as much oil as possible with concomitantly high saving of the arising PR. This means, higher temperatures might have separated more oil but would have damaged sensitive ingredients of the PR like antioxidants. This feature originated different extraction efficiencies of approximately 50%. For all seeds the same pressing process was used. The ground and sieved black currant PRs contained 16.5% or 25.7% oil depending on the batch of black currant seeds (Table 1). Unprocessed black currant seeds contained 29.3% oil. The high variation in the oil contents of the PRs was possibly due to variations in the oil content from different seed batches (Ruiz del Castillo et al., 2002). The FA distribution in black currant PRs showed a high level of linoleic acid (C18:2, *n*-6, 48%), α -linolenic (C18:3,

n-3, 15%) and γ -linolenic acid (C18:3, *n*-6, 13%) (Table 2) comparable with the results reported by Stránský, Zarevúčka, and Wimmer (2005). The predominating FA in the PRs investigated was linoleic acid with a mean of $41 \pm 7\%$, followed by α -linolenic acid with $29 \pm 9\%$ and oleic acid (C18:1, *n*-9) with $17 \pm 5\%$. Only black currant contained appreciable amounts of γ -linolenic acid, with approximately $13 \pm 0\%$. All other FAs consisted of less than 10% in all PRs. According to Ruiz del Castillo et al. (2002), higher contents of γ -linolenic acid were negatively correlated with α -linolenic acid indicating a competitive inhibition of the $\Delta 6$ and $\Delta 15$ desaturases. Both of these enzymes are responsible for the conversion of the precursor linoleic acid into α -linolenic acid or γ -linolenic acid, respectively. The sum of C18:3 (*n*-3 + *n*-6) of all PRs ranged between 23% (rose hip) and 43% (cranberry) with an overall mean of $26 \pm 10\%$. There was a large variation in the ratio of α -linolenic acid to γ -linolenic acid. The PR of each of the berry seeds contained α -linolenic acid, while γ -linolenic acid was only detected in black currant. Among the species, the physiologically important stearidonic acid (C18:4, *n*-3) was only found in black currant (2.9%). Stearidonic acid mainly exists in the seed oils of black currant, evening primrose and borage (Ursin, 2003) and is a precursor of EPA in humans (Miles, Banerjee, & Calder, 2004). No differences were observed in the FA distribution of whole black currant seeds and black currant PRs, processing does not change the FA distribution.

In the PRs, the content of saturated fatty acids amounted for less than 10% of the total FAME detected. The content of monounsaturated fatty acids (MUFA) varied between 13% (black currant I) and 24% of total FAME (bilberry and cranberry), while the polyunsaturated fatty acids (PUFA) content varied between 64.2% (bilberry) and 79.2% of total FAME (black currant II). More than 94% of the detected FAs were long-chain fatty acids (C15–C24) while medium-chain fatty acids (C11–C14) represented less than 0.1% of total FAME. Nutrition societies recommend an *n*-6/*n*-3 FA ratio of 5:1 for a healthy diet (DACH, 2002). All tested berry PRs demonstrated a lower ratio (<3.6:1) with cranberry having the lowest ratio of 0.7:1. Therefore PRs can improve the average human diet in western countries, since the *n*-6/*n*-3 ratio in most industrialized countries is generally as high as 15:1 (Simopoulos, 2002).

3.2. Tocopherols and tocotrienols

Differences between batches within one species may result from environmental influences, e.g., geographical location or harvesting time (Kallio, Yang, & Peippo, 2002a). Black currant PR I and II contained not only different tocopherol concentrations, but also different tocopherol profiles (Table 1). The largest amounts of tocopherols were found in elder and black currant PR. The main tocopherol isomers in the species tested were α - and γ -tocopherol, whereas α -tocopherol was the principle isomer in black currant I

Table 1

Tocopherol and tocotrienol concentrations in various berry seed press residues given as means of duplicate or triplicate measurements [mg/100 g residue]

PR sample	Tocopherol [mg/100 g PR]				Tocotrienol [mg/100 g PR]				Oil content [%]	Tocopherol	
	α	β	γ	δ	α	β	γ	δ		[mg/100 g oil of PR]	Tocotrienol
Bilberry	0.89	0.04	0.33	–	0.24	–	1.57	–	5.2	24.6	33.0
Cranberry	0.87	0.16	0.15	–	1.01	–	3.92	–	5.6	21.0	93.0
Rose hip	0.78	–	0.87	–	–	–	–	–	4.0	40.7	–
Strawberry	0.49	–	0.91	–	–	–	–	–	6.2	22.7	–
Elder	0.96	0.31	12.44	0.16	0.89	–	–	–	12.0	115.3	7.1
Black currant I	8.94	0.34	7.99	0.26	–	–	–	–	16.5	106.6	–
Black currant II	2.82	0.14	5.97	0.18	–	–	0.12	–	25.7	35.5	0.5

PR: Berry seed press residue.

Table 2

Fatty acid distribution of various seed press residues given as means of duplicate or triplicate measurements [mol% of FAME]

Fatty acid	Bilberry	Cranberry	Rose hip	Strawberry	Black currant I	Black currant II	Black currant (whole seeds)
C12:0	0.01	n.d.	0.04	0.03	0.01	n.d.	0.01
C14:0	0.06	0.04	0.06	0.06	0.04	0.04	0.03
C16:0	5.18	1.10	3.28	5.34	6.26	6.11	6.16
C18:0	1.30	0.23	1.98	1.68	1.26	1.31	1.17
C20:0	0.19	0.04	0.91	0.59	0.08	0.09	0.11
C21:0	0.15	n.d.	0.23	0.19	0.03	n.d.	n.d.
C22:0	0.16	n.d.	0.29	0.27	0.04	n.d.	0.06
C24:0	0.14	n.d.	0.24	0.22	n.d.	n.d.	0.06
C18:1 _{n-9}	22.12	22.52	14.84	19.91	10.79	11.00	11.09
C18:1 _{n-7}	0.67	0.64	0.49	0.63	0.77	0.75	0.76
C20:1 _{n-9}	0.22	0.25	0.51	0.29	0.76	0.89	0.90
C18:2 _{n-6}	33.59	31.02	47.68	36.19	47.91	47.86	47.78
C20:2 _{n-6}	0.04	0.23	0.15	0.06	0.23	0.26	0.27
C18:3 _{n-3}	0.07	n.d.	n.d.	0.02	13.03	12.94	12.55
C18:3 _{n-3}	29.84	43.54	23.09	28.18	14.58	15.13	14.75
C20:3 _{n-3}	0.19	0.06	0.99	0.16	n.d.	0.03	n.d.
C18:4 _{n-3}	0.04	n.d.	0.03	0.04	2.85	2.94	2.71
Minor FA	6.03	0.33	5.19	6.14	1.36	0.65	1.59
SFA	7.25	1.78	7.57	8.48	7.81	7.63	7.60
MUFA	23.55	23.41	16.42	21.53	12.46	12.64	13.01
PUFA	64.16	74.85	72.21	64.96	78.62	79.18	77.79
LCFA	94.85	99.79	96.07	94.87	98.85	99.59	98.36
n-3	30.01	43.60	23.20	28.31	17.43	18.09	17.46
n-6	34.75	31.25	49.51	37.29	61.96	61.81	60.59
n-9	22.47	22.77	15.47	20.38	11.55	11.89	11.99
n-6/n-3	1.16	0.72	2.13	1.33	3.56	3.42	3.47

n.d.: Below detection limit (values <0.001% of FAME).

FAME: Fatty acid methyl esters; FA: Fatty acids; SFA: Saturated fatty acids, includes not separately listed SFA; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; LCFA: Long-chain fatty acids (C15–C24), the value includes minor fatty acids determined but not separately listed.

PR, and γ -tocopherol primarily in elder and black currant II PR. Tocotrienols were detected in high concentrations in cranberry and bilberry PR where they dominated over tocopherols. Nevertheless, compared to the total tocopherol concentrations of the other species they exhibited lower total tocotrienol concentrations. Neither rose hip nor strawberry PRs contained measurable amounts of tocotrienols. The tocopherol and tocotrienol related to the remaining oil concentrations within the PR demonstrated high tocopherol concentrations, especially in elder and black currant I (115 and 107 mg/100 g oil, respectively). The tocopherol concentration of the PR of elder and black currant was comparable to other tocopherol-rich commercial seed oils namely maize and soybean oil (162 and 180 mg/100 g oil, respectively) (Tuberoso, Kowalczyk, Sarritzu, & Cabras, 2007).

Statistical analysis basing on the tocopherols and FAs in PR revealed a positive correlation between γ -tocopherol and the sum of n-6 FAs (Table 3). For α -tocopherol this relation was not significant. The n-3 FAs demonstrated a negative but insignificant association to the PR α - and γ -tocopherol concentrations. In contrast, n-3 FAs and α - and γ -tocotrienols correlated positively. These results support a positive correlation of the n-6/n-3 ratio and the α - and γ -tocopherol concentrations. Comparing the coefficients of correlation of tocotrienol and tocopherol concentrations towards

the n-3 FAs, n-6 FAs, n-9 FAs, or n-6/n-3 ratio, the coefficients exhibited contrary behaviour, i.e., for example, tocopherols correlating negatively to n-3 FAs are associated with tocotrienols correlating positively to n-3 FAs and *vice versa*. Positive associations of PUFAs could be verified to β -tocopherol with $R = 0.734$, $p = 0.097$, and γ -tocopherol with $R = 0.740$, $p = 0.092$ (α -tocopherol: $R = 0.622$, $p = 0.187$). In contrast, MUFAs were basically negatively associated to α - and γ -tocopherols.

3.3. Phytosterols and carotenoids

Only moderate amounts of phytosterols (sitosterol: 76.6 mg/100 g, campesterol: 6.24 mg/100 g) and carotenoids (lutein: 0.778 mg/100 g, α -carotene: 0.537 mg/100 g, (*all-E*)- β -carotene: 0.223 mg/100 g, (9Z)- β -carotene: 0.042 mg/100 g) were detected in black currant I PR. In comparison, carotenoid-rich plants such as spinach or carrots contain approximately 17 and 16 mg carotenoids/100 g, respectively (Müller, 1997). Saito et al. (2006) reported a positive health effect in mild hypercholesterolemic subjects when a mayonnaise enriched with 0.3 g/d phytosterol esters was consumed for 4 weeks. The phytosterol concentration in the black currant PR was generally too low to reduce serum low-density lipoprotein cholesterol. Phytosterol concentrations of sea

Table 3Correlation coefficients of tocopherol or tocotrienol concentrations and fatty acid parameters of seed press residues^a

	Tocopherol				Tocotrienol			
	α		γ		α		γ	
	R	P	R	P	R	P	R	P
MUFA	-0.701	0.121	-0.881	0.020	0.611	0.198	0.667	0.148
PUFA	0.622	0.187	0.740	0.092	0.043	0.935	-0.041	0.938
n-3	-0.581	0.227	-0.758	0.081	0.906	0.013	0.908	0.012
n-6	0.719	0.107	0.904	0.013	-0.641	0.170	-0.682	0.135
n-9	-0.701	0.121	-0.880	0.021	0.638	0.173	0.693	0.127
n-6/n-3	0.764	0.077	0.928	0.006	-0.642	0.169	-0.677	0.140

^a Press residues of bilberry, cranberry, rose hip, strawberry, and black currant I and II were used for correlation analyses.

buckthorn (*Hippophae rhamnoides* L.) were reported to be in the same concentration range (1.2–1.8 mg/g whole seeds) as of the black currant PR (Yang, Karlsson, Oksman, & Kallio, 2001). The black currant PR phytosterol profile was dominated by the sitosterol fraction with 92.5% of total phytosterols. The sitosterol fraction was lower in blueberry and lingonberry (85 and 80% in free sterols, 57 and 42% in esterified sterols, respectively), and also the campesterol fraction (7 and 6% in free sterols, 5 and 3% in esterified sterols, respectively) was lower in these two berries (Yang et al., 2003). Our analysis quantified the most dominating phytosterol peaks, leading to different relative portions compared to the literature. In general, phytosterols in seeds range between 22 and 714 mg/100 g seeds (Pegel, 1997).

3.4. Antioxidant parameters (TEACs, total phenols as GAE, vitamin C)

The TEAC II values varied between 5.8 (bilberry) and 84.4 (elder) $\mu\text{mol}/100\text{ g}$ (Fig. 1). Black currant showed higher TEAC II values (74.7 and 67.2 $\mu\text{mol}/100\text{ g}$) compared to the other species PRs tested. TEAC III values ranged between 19.5 and 83.9 $\mu\text{mol}/\text{g}$ and thus were approximately 150 times higher than the TEAC II values. Both TEAC assays measured the ability of berry seed PR extracts to reduce *in vitro* formed radicals (Böhm & Schlesier, 2004). The TEAC value of an extract is the product of a sum of antioxidant compounds that will depend on the solvent used to extract the test matrix (Schlesier et al., 2002). TEAC II measurements performed using hexane comprised tocopherols and lipophilic compounds that are not included when extracting with water (TEAC III) that includes only hydrophilic compounds (Schlesier et al., 2002). The latter are obviously more abundant in the PRs than lipophilic compounds. These characteristic features of the TEAC tests were reflected in the correlation coefficients of the experimental results: the TEAC II values of the PRs correlated significantly with the PR tocopherols ($R = 0.893$, $p = 0.017$). TEAC III demonstrated no significant correlation with the total tocopherol content ($R = 0.033$, $p = 0.945$). TEAC II values ($R = -0.371$, $p = 0.469$), vitamin C ($R = 0.533$, $p = 0.173$) or GAE ($R = 0.231$, $p = 0.582$). One group of hydrophilic compounds responsible for high TEAC III values can be flavonoids that exist in the PRs. Anthocyanins as delphinidin and cyanidin bound as rutinoides and some glucosides are the major forms found within the black currant PR, where the whole berry showed a similar composition like the seed (Kapasakalidis, Rastall, & Gordon, 2006; Lu & Foo, 2003). In addition black currant berries contained high concentrations of proanthocyanidines, mostly found as procyanidin and prodelphinidin polymers (Wu,

Gu, Prior, & McKay, 2004). Commonly utilized methods are incapable of extracting these compounds from the sample and proanthocyanidines were not determined in this work. Most of the antioxidant capacity determinations revealed higher antioxidant capacity for elder berries than for black currant berries (Wu et al., 2004). Our results of TEAC III and GAE for the PRs in part verified these differences (Figs. 1 and 2). The comparison of TEAC III values of the PRs (19.5–83.9 $\mu\text{mol}/\text{g}$) to other fruits like prunes (14.8 $\mu\text{mol}/\text{g}$) and figs (5.02 $\mu\text{mol}/\text{g}$) revealed that PRs contained significant levels of antioxidants similar to strong antioxidant herbs (rosemary, 43.95 $\mu\text{mol}/\text{g}$) (Pellegrini et al., 2006).

As demonstrated in black currant, there is an enormous variation in the GAE within one species (Fig. 2). The analysis of four different *Ribes nigrum* cultivars revealed GAE-concentrations between 0.72 and 1.16 g/100 g of berries (Costantino, Albasini, Rastelli, & Benvenuti, 1992). Vinson, Su, Zubik, and Bose (2001) quantified 0.256 g/100 g in fresh bilberry, and 0.221 g/100 g in bilberry PR. Grapes known as a rich source of polyphenols contained a GAE of 0.294 g/100 g (Vinson et al., 2001). Generally, GAE represents the total phenolic content of a sample and utilizes gallic acid as a standard phenol which exhibits the strongest antioxidant capacity in several test systems when compared with other antioxidants like uric acid, Trolox®, and ascorbic acid (Schlesier et al., 2002; Stratil, Klejdus, & Kubán, 2006). GAE was associated with the vitamin C ($R = 0.661$, $p = 0.075$), the TEAC II values ($R = -0.755$, $p = 0.140$), and the tocopherol concentrations ($R = -0.783$, $p = 0.066$) in the PRs. Thus, PRs with a high tocopherol content and TEAC II values possessed low levels of vitamin C and GAE, and vice versa. Vitamin C concentrations varied between 15.0 mg/100 g (strawberry) and 68.6 mg/100 g (bilberry), and were observed in a similar range as fresh black currant juice (39.2 mg/100 mL, Young et al., 1999). The Folin-Ciocalteu reagent reacts also with ascorbic acid, leading to the correlation between vitamin C and GAE (Stratil et al., 2006).

3.5. Fibre

The mean total fibre content in the tested PRs was 60 mg/100 g, being highest in rose hip (71.3 mg/100 g) and lowest in black currant II (48.2 mg/100 g) and cranberry (48.5 mg/100 g). Black currant I, bilberry, strawberry and elder contained 58.6–59.4 mg/100 g. For a better characterization of the fibre, a detergent fibre analysis was performed with black currant PR. The NDF (insoluble fibres) was 42.5 g/100 g, the soluble fibres were 57.5 g/100 g and the ADF was 18.5 g/100 g. The insoluble fibre consisted of 8.2 g/100 g lignin (cutin was not determined and removed from the

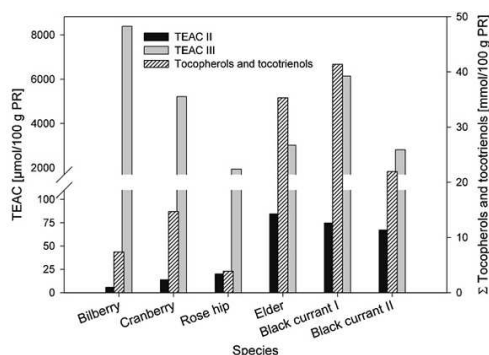


Fig. 1. Trolox equivalent antioxidant capacities (TEAC II and III) and total tocopherol concentrations of various species of berry seed press residues (PR) given as means of duplicate or triplicate measurements.

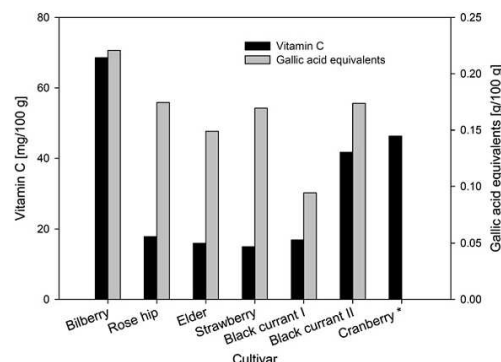


Fig. 2. Gallic acid equivalent (GAE) and vitamin C concentrations in various berry seed press residues (PR) given as means of duplicate or triplicate measurements * GAE not determined.

ADL fraction), 10.3 g/100 g cellulose, and 24.0 g/100 g hemicellulose. Nawirska and Kwaśniewska (2005) determined 91% total fibre in the dry matter of black currant pomace after fruit processing. The fresh fruit pomace and the seed PR had same levels of hemicellulose (25% and 24%, respectively) and cellulose (12% and 10%, respectively) suggesting that fruit pomace and PRs had similar fibre content. Both matrices had a dry matter content of about 93%. The lignin content in fruit pomace was 73.8 g/100 g of dry matter which was much greater compared to the seed residue with 8.2 g/100 g (Nawirska & Kwaśniewska 2005).

3.6. Protein and amino acids

The protein is a quantitatively important fraction in the PRs (Table 4). The most frequent AA was glutamine (17%), followed by aspartic acid (9%) and arginine (8%).

Based on the WHO AA requirements, in the tested matrices (black currant PR I, II, and whole seeds) tryptophan was the limiting AA for adults (AAR 2.0/2.2/2.2). For the 10–12-year-old adolescents lysine was the limiting AA in each matrix (AAR 1.1), and additionally tryptophan in black currant PR I (AAR 1.1). The ratio of essential AAs to whole egg AAs estimates the BQ. Compared to whole egg, the lower BQ values were due to the imbalance between the concentrations of the essential AAs in the PR and seeds. The EAAI is another classification parameter for protein which is closely associated to the BQ. The EAAI reflected the same quality as the BQ values (Table 4). The protein of the black currant seeds is of good quality, evident by the AAR of >1 for all the essential AAs. These AAs features evaluated do not take protein digestibility into consideration which is thought to be as important as the protein quality, but which was not investigated in this study.

3.7. Dry matter, ash content, and elements

PR of black currant was rich in dry matter (93.5%) of which 3.96% was ash containing the elements K (6.99 mg/g) > P

Table 4
Content of amino acids, crude protein and parameters of protein quality* in black currant seed press residues and whole seeds given as means of duplicate measurements [g/16 g N]

		Black currant I	Black currant II	Black currant (whole seeds)
Amino acid [g/16 g N]	Cys	2.3	2.7	2.8
	Asp	8.6	9.1	9.4
	Met	1.9	2.1	1.7
	Thr	3.6	4.0	3.3
	Ser	3.9	3.8	3.8
	Glu	17.4	17.8	17.8
	Gly	4.8	5.0	5.2
	Ala	4.0	3.9	4.2
	Val	2.9	2.9	3.1
	Ile	4.5	4.6	4.9
	Leu	5.6	5.8	6.1
	Tyr	2.2	2.3	2.5
	Phe	3.8	3.9	4.1
	His	3.5	3.5	3.8
	Lys	4.4	4.7	5.0
	Arg	7.5	8.0	8.3
	Pro	3.3	3.7	3.8
	Hypro	0.0	0.0	0.0
	Trp	1.1	1.1	1.1
	Sum	85.3	88.9	90.9
Crude protein [mg/100 g residue]		21.5	22.5	19.4
EAAI		0.72	0.75	0.76
BQ*		63	67	68

EAAI: Essential amino acid-index.

BQ: Biological quality (approximated).

* Ratio of essential amino acids to whole egg amino acids

(6.12 mg/g) > Ca (4.83 mg/g) > S (2.51 mg/g) > Mg (1.89 mg/g). Moderate concentrations of the following elements were detected: Fe at 0.19 mg/g, and Na, Al, Zn, Mn, and Cu at <0.1 mg/g. The concentrations of As, Ba, Cd, Co, Cr, Ni, Mo, Pb, Se, Sr, Ti, and V were <0.01 mg/g.

The PRs had a high concentration of iron relative to other plants. In comparison, oat and wheat flour contained 0.023 and 0.011 mg/g, respectively (Cook, Reddy, Burri, Juillerat, & Hurrell, 1997). The concentration of iron in the PR may be elevated due to abrasions of the mill during processing.

4. Conclusions

The PRs tested contained moderate to high concentrations of tocopherols and some tocotrienols. In addition, there was a favourable $n-6/n-3$ ratio and a high PUFA content of >65% with linoleic acid predominating (>34%). The content of stearidonic acid in black currant was conspicuously high.

Some of the PRs were distinguished by moderate to high concentrations of GAE, comparable with fresh grapes for example. The water soluble fraction of the PRs showed high TEAC values that were at least as high as that of herbs. These results need to be put into perspective, because PRs cannot be consumed in the same amount or manner as grapes or apples. The protein quality of black currant seeds was of good grade, and contained all essential AAs in fairly balanced proportions. Phytosterols and carotenoids were present only in trace amounts.

In summary, berry PRs contained several components with health-promoting attributes. Future processing may enable us to convert these residues into edible products so their potential can be utilized.

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MANUSCRIPT II (ACCEPTED)

Black currant seed press residue increases tocopherol concentrations in serum
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in humans

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Blackcurrant seed press residue increases tocopherol concentrations in serum and stool whilst biomarkers in stool and urine indicate increased oxidative stress in human subjects

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Berry seeds are a tocopherol-rich by-product of fruit processing without specific commercial value. In a human intervention study, the physiological impact of blackcurrant seed press residue (PR) was tested. Thirty-six women (aged 24 ± 3 years; twenty non-smokers, sixteen smokers) consumed 250 g bread/d containing 8% PR for a period of 4 weeks (period 3). Comparatively, a control bread without PR (250 g/d) was tested (period 2) and baseline data were obtained (period 1). Blood, stool and 24 h urine were collected during a 5 d standardised diet within each period. Tocopherol and Fe intakes were calculated from food intake. In serum, tocopherol concentration and Fe parameters were determined. In urine, oxidative stress markers 8-oxo-2'-deoxyguanosine, 8-iso-PGF_{2α} and inflammatory response marker 15-keto-dihydro-PGF_{2α} were analysed. Stool tocopherol concentration, genotoxicity of faecal water (comet assay) and antioxidant capacity of stool (aromatic hydroxylation of salicylic acid) were determined. Fe and total tocopherol intake, total tocopherol concentrations in serum and stool, and genotoxicity of faecal water increased with PR bread consumption ($P < 0.05$). The antioxidant capacity of stool decreased between baseline and intervention, expressed by increased formation of 2,3- and 2,5-dihydroxybenzoic acid *in vitro* ($P < 0.05$). In smokers, 8-oxo-2'-deoxyguanosine increased with PR consumption ($P < 0.05$). Prostate concentrations were unaffected by PR bread consumption. In summary, the intake of bread containing blackcurrant PR for 4 weeks increased serum and stool total tocopherol concentrations. However, various biomarkers indicated increased oxidative stress, suggesting that consumption of ground berry seed may not be of advantage.

Blackcurrants: Tocopherol: Comet assay: Antioxidant capacity in stool

Berry seeds, a by-product of juice fabrication with promising health benefits, are currently an unutilised nutritional resource. The seeds are rich in PUFA, tocopherols, fibre and contain polyphenols^(1,2). Most of these ingredients are known to be associated with oxidation processes.

Oxidation of cell compounds such as DNA, lipids or proteins is described as a promotion of atherogenesis and carcinogenesis^(3,4). Evidence for the correlation between oxidative DNA damage and human degenerative diseases such as CHD has previously been provided⁽⁵⁾. It is presumed that the prevalence of CHD and also of carcinogenesis is associated with tocopherol in serum or with tocopherol intake, although results are ambivalent. However, it has been shown that CHD patients are characterised by a high serum α : γ -tocopherol ratio⁽⁶⁾ and decreased γ -tocopherol serum concentration compared with controls⁽⁷⁾. In addition, the deficiency of α -tocopherol in lipoproteins was not associated with atherosclerosis⁽⁸⁾. The results of the few studies dealing with fruit or berry consumption show inconclusive evidence of the effects on DNA damage in leucocytes⁽⁹⁾.

The findings of the Nurses' Health Study deny an association of vitamin E supplementation and the risk of colon cancer⁽¹⁰⁾.

Studies concerning berry consumption basically focused on whole berries or berry juices and extracts, rather than on the seeds and their potential^(11–13). Thus, the present human intervention study was conducted in order to correct omission. Different markers were used to evaluate the physiological and health-beneficial impact of blackcurrant press residue (PR). The most likely effects are probably due to the antioxidant potential of substances in PR. The matrices stool, urine and serum were included. Stool is a very complex and individual matrix, with various substance groups that may affect the antioxidant capacity. Examining one single substance is not always consequential, since synergies and correlations between different substances are very likely. The influence of PR on antioxidant capacity of stool can be evaluated by means of the aromatic hydroxylation of salicylic acid⁽¹⁴⁾. Characterising the DNA-damaging potential in stool can be performed using microgel electrophoresis with faecal water-incubated cells^(15,16). Genotoxicity of faecal water indicates

Abbreviations: DHBA, dihydroxy benzoic acid; 8-oxodG, 8-oxo-2'-deoxyguanosine; PI, period 1; PII, period 2; PIII, period 3; PR, press residue.

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the exposition of colon cells towards genotoxic compounds that lead to increased DNA damage and a risk of colon cancer.

Further, in the present study the influence of PR on oxidative DNA damage of the whole body was measured using the biomarker 8-oxo-2'-deoxyguanosine (8-oxodG) in urine. 8-OxodG, a product of DNA repair excreted in urine, is generated by oxidative stress causing the transformation of the nucleoside guanosine⁽¹⁷⁾. The excreted amount of 8-oxodG correlates with the extent of oxidative stress in an individual. Oxidative stress occurs as a result of different factors such as the presence of disease⁽¹⁸⁾, smoking⁽¹⁹⁾, physical exercise in untrained subjects⁽²⁰⁾ and enzymic activities such as that of glutathione-S-transferase⁽²¹⁾. On the other hand, there are factors that can contribute to decrease the oxidative stress. These factors include a high consumption of fruits and vegetables containing significant concentrations of polyphenols and other substances⁽²²⁾, moderate physical activity⁽²³⁾ or the use of hormonal contraceptives⁽²⁴⁾.

The aim of the study was to test whether serum total tocopherol concentrations and parameters linked to oxidative stress are influenced by the intake of blackcurrant PR in human subjects. Secondly, effects of lifestyle parameters that are associated with oxidative stress such as smoking or the use of hormonal contraceptives were also considered.

Experimental methods

Test substance, diets and experimental design

Berry seed PR contains significant amounts of tocopherols (α , 6.56 $\mu\text{mol}/100\text{ g}$; β , 0.32 $\mu\text{mol}/100\text{ g}$; γ , 14.3 $\mu\text{mol}/100\text{ g}$; δ , 0.44 $\mu\text{mol}/100\text{ g}$) as well as γ -tocotrienol (0.29 $\mu\text{mol}/100\text{ g}$) according to Helbig *et al.*⁽¹⁾. The PR has a gallic acid equivalent of 0.17 g/100 g, and a trolox equivalent of 2.82 mmol/100 g in a hydrophilic- and 67.2 $\mu\text{mol}/100\text{ g}$ in a lipophilic solvent. As described previously, the PR contains 25.7 g fat, 48.2 g fibre, 22.5 g crude protein and 19 mg Fe/100 g⁽¹⁾. Inositol hexaphosphate (phytic acid) concentration of the applied PR was 0.63 mmol/100 g and no intermediate inositol phosphates were quantified. β -Carotene and plant sterols were not present in significant amounts⁽¹⁾. The concentrations of yeasts, mould and bacteria measured (, unpublished results) were all below the upper levels considered safe for ground grain products with reference to the values released by the German Society of Hygiene and Microbiology (Regulation (EU) 2073/2005).

Blackcurrant seeds were pressed, ground and sieved to obtain an acceptable mouth feeling. The PR was baked into bread at a maximum dose of 8 % that still allowed the correct preparation of the dough. No significant losses of tocopherol were verified by the baking process. The daily amount of 250 g test bread that had to be consumed by the participants contained 20 g PR. The control bread contained no PR, but was otherwise identical to the test bread. Here too, an amount of 250 g/d had to be ingested. The bread was made using wheat flour, rye flour, crushed rye grain, oat and flax seeds. The total tocopherol concentrations in the control and test bread were 3.86 and 6.32 $\mu\text{mol}/100\text{ g}$, respectively. The sum of inositol hexa-, penta-, tetra- and triphosphate concentration was 0.50 mmol/100 g for control bread and 0.89 mmol/100 g for test bread.

The intervention study comprised three periods: a 5 d baseline period consisting of a normal diet without intervention (period 1; PI) for obtaining baseline data. It was followed by a 4-week period with an intake of control bread (period 2; PII) which was then substituted by test bread in the next 4-week intervention period (period 3; PIII). During baseline and the last 5 d of PII and PIII a standardised diet was administered. At the same time, complete stool and 24 h urine were collected for 3 d as well as one blood sample that was taken by authorised nurses in the morning after overnight fasting. During the standardised diets the consumption of the respective bread was continued. Participants were instructed to eat normally, not to go on a diet and to try to include the bread in their eating habits without increasing their daily energy intake during the study. Except for the control and test bread in the respective periods, the 5 d standardised diet packages were identical between the three study periods and daily included one warm meal, fruits, vegetables, dairy products, bread-spreads and drinks. Participants were free to consume everything provided in the food package, though they were advised to resemble their food intake at each standardised diet period. The uneaten daily portions had to be returned for quantification of the actual food consumption.

If not described otherwise, nutrient intake was calculated using PRODI[®] 5.4 software (Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, Germany). At the start and the end of the study, a bioelectrical impedance analysis (BIA) was conducted for purposes of monitoring the physical constitution of the participants (BIA 2000-C; Data Input, Darmstadt, Germany).

Subjects

Initially, we recruited twenty voluntary female smokers and twenty female non-smokers via placards and newspaper advertisements. Subsequently, four participants from the group of smokers dropped out of the study for individual reasons (for example, illness, lack of time). Thus, results presented are based on a total of thirty-six participants; twenty non-smokers and sixteen smokers. Smokers were defined by a consumption of at least five cigarettes/d. The mean cigarette consumption was 9 (SD 5; range 3–20) cigarettes/d. Participants were allowed to smoke before blood withdrawals. Moreover, twenty-two participants (ten smokers and twelve non-smokers) used a hormonal contraceptive, whereas fourteen participants (six smokers, eight non-smokers) did not. Criteria for including subjects into the study were not doing serious sports, no diagnosed diseases, and no use of nutritional supplements 2 weeks before and during the study. Participants were aged 24 (SD 3; range 18–33) years. The mean BMI constituted 23.3 (SD 3.7; range 17.9–32.8) kg/m² with a body size of 170 (SD 7; range 157–186) cm and a body weight of 67 (SD 11; range 53–97) kg. All test individuals were informed as regards the purpose, the course and their responsibilities during the study before they gave their informed written consent. The study was approved by the Ethics Committee of the Medical Faculty of the Friedrich Schiller University of Jena, Germany (approval no. 1485-01/05).

Preparation of human samples

The complete, fresh stool was collected in plastic bags, transported to the laboratory and immediately stored at -20°C .

After receiving all stool samples, stool was defrosted, homogenised and subsequently lyophilised, separately for each participant and period. For gaining faecal water, samples of the homogenised, defrosted stool were weighed into polyethylene tubes (Beckman Coulter, Munich, Germany), and centrifuged for 4 h at 21000 rpm and 4°C (Beckman J2-21). The supernatant fraction representing the faecal water was stored in cryogenic tubes (Roth, Karlsruhe, Germany) at -20°C. Samples of urine taken from the daily samples were stored at -20°C. The defrosted samples were mixed according to the proportion of the daily excreted urine volume, separately for each participant and period. Blood was collected into serum tubes (BD Vacutainer Systems, Heidelberg, Germany) and centrifuged for 20 min at 4000 rpm. Serum obtained was frozen at -20°C until analysis.

Tocopherols

According to Kuhnt *et al.* lyophilised food and stool were added with ascorbic acid (Fluka, Buchs, Switzerland) and saponified⁽²⁵⁾. Extraction was carried out using *n*-hexane containing 2,6-di-*tert*-butyl-*p*-kresol (BHT) (VWR, Leuven, Belgium and Fluka, Buchs, Switzerland, respectively). With each batch executed, a reference milk powder of defined tocopherol concentration was analysed in parallel (BCR-421, Report EUR 18320 EN; Promochem, Wesel, Germany). Serum samples were prepared with ethanol-BHT solution (ethanol: Roth, Karlsruhe, Germany), shaken and extracted using *n*-hexane containing BHT. Extracts were measured by means of HPLC-fluorescence (Shimadzu, Tokyo, Japan; Nucleosil 100 NH₂ column, 250 × 4 mm; Macherey & Nagel, Dueren, Germany). Analysed α-, β-, γ- and δ-tocopherol concentrations were summed up to total tocopherol (tocopherol standards; Calbiochem, Darmstadt, Germany).

Hydroxylation products of salicylic acid in stool (antioxidant capacity of stool)

According to the method of Owen *et al.* phosphate buffer (100 mM, KH₂PO₄ and K₂HPO₄; Merck, Darmstadt, Germany) was prepared with EDTA (500 μM; Roth, Karlsruhe, Germany), FeCl₃ (iron 50 μM; Merck, Darmstadt, Germany) and salicylic acid (2 mM; Merck, Darmstadt, Germany) in HPLC-grade water (pH 6.5)⁽¹⁴⁾. Fe³⁺ and EDTA are added to the test system to support the generation of hydroxyl radicals. These radicals oxidise the salicylic acid to 2,3- and 2,5-dihydroxy benzoic acid (DHBA) and catechol which can all be quantified. Lyophilised stool (0.1 g) was mixed with 10 ml phosphate buffer and incubated in an orbital shaker at 200 rpm for 18 h at 37°C. After centrifugation (4000 rpm, 40 min, Rotina 46; Hettich Zentrifugen, Tuttlingen, Germany), the supernatant fraction was filtered using a sterile, pyrogen-free filter (0.2 μm, Chromafil; Macherey & Nagel, Dueren, Germany) and measured by means of HPLC-UV (diphenols 325 nm, catechol 278 nm; column: Hypersil C18 ODS II, 250 × 4 mm; Agilent, Waldbronn, Germany). Unlike Owen *et al.* for gradient elution the mobile phase consisted of methanol (VWR, Leuven, Belgium) and ammonium acetate buffer (Merck, Darmstadt, Germany; pH 3-6 with acetic acid, Roth, Karlsruhe, Germany)⁽¹⁴⁾. Standard curves obtained

from catechol, 2,5-DHBA and 2,3-DHBA (Sigma-Aldrich, Steinheim, Germany) were utilised for calculating the results.

Iron parameters

For the determination of serum Fe parameters, the Abbott Architect c8000 analyser and the corresponding test kits were used according to the manufacturer's instructions (Abbott, Wiesbaden, Germany and Abbott Laboratories, Baar, Switzerland). The ferritin assay was performed with the chemiluminescent microparticle immunoassay. Serum Fe was analysed colorimetrically by means of the Fe assay; the transferrin was analysed using an immunoturbidimetric assay.

Fe concentrations in test and control bread as well as in the stool were analysed via ICP-OES (Spectroflame, Spectro, Kleve, Germany) according to DIN 38406 (E22). The stool sample was reduced to ash and mixed with water and HCl (Roth, Karlsruhe, Germany), then heated and filtrated. Before measuring, the sample was acidified with ultrapure HNO₃ (Roth, Karlsruhe, Germany) to a final concentration of 2 % of the sample and diluted at a ratio of 1:2 with ultrapure water. Unlike described in the used DIN method, the calibration range was adjusted according to the expected concentrations (0.005–5.0 mg/l, multi-element standard Merck IV; Merck, Darmstadt, Germany).

The Fe intake from the foods during the standardised diet that were consumed besides the breads was calculated using the PRODI[®] 5.4 software (Wissenschaftliche Verlagsgesellschaft mbH).

Cyto- and genotoxicity of faecal water

Cyto- and genotoxicity of faecal water were tested using HT29 clone cells treated with 10 % faecal water for 30 min at 37°C. The determination of cell viability before and after incubation with faecal water was accomplished via the Trypan Blue exclusion⁽²⁶⁾. Analyses on genotoxicity of the faecal water were performed using single-cell micro-gel-electrophoresis (comet assay) measuring tail intensity according to Oberreuther-Moschner *et al.*⁽²⁷⁾.

Urinary 8-oxo-2'-deoxyguanosine

The urine samples were purified on a C18 EC column (Macherey & Nagel, Dueren, Germany). 8-OxodG was eluted with methanol (VWR, Leuven, Belgium), concentrated under a stream of N₂, resolved in HPLC-grade water and measured by means of HPLC-electrochemical detection (Shimadzu, Tokyo, Japan; column: Hypersil C18 ODS II, 250 × 4 mm; Agilent, Waldbronn, Germany) according to Kuhnt *et al.*⁽²⁵⁾. For calibration purposes, 8-oxodG was purchased from Sigma-Aldrich (Munich, Germany).

F₂-isoprostanes and prostaglandin F_{2α} metabolite

8-Iso-PGF_{2α}, a standard marker of oxidative stress, and 15-keto-dihydro-PGF_{2α}, a reliable marker of inflammatory response formed through the cyclo-oxygenase pathway, were analysed in urine samples using two separate RIA, as described previously^(28,29).

Statistical analysis

Statistical analysis was carried out using the SPSS 14.0 software package (SPSS, Inc., Chicago, IL, USA). Results were tested for normal distribution by means of the Kolmogorov–Smirnov test. If not described differently, significance was checked using repeated-measures ANOVA. The one-sided Mann–Whitney *U* test was used to compare subgroups. For correlation analysis, the Pearson's correlation coefficient was determined. Values were referred to as significant at $P < 0.05$.

Results

The physical constitution of subjects measured assessed via bioelectrical impedance analysis remained unchanged during the study. The mean energy intake with the standardised diets was 7350 (SD 811) kJ/d (baseline). This value significantly increased during control (8000 (SD 1063) kJ/d) and test bread consumption (7771 (SD 1185) kJ/d; $P < 0.05$). The results were only split into subgroups of smokers, non-smokers as well as users and non-users of hormonal contraceptives when regarded as necessary, i.e. if there were any significant changes apparent between the periods.

Tocopherols

The total tocopherol intake at baseline, control and intervention was 42.0 (SD 5.9), 43.3 (SD 6.7) and 51.4 (SD 8.2) $\mu\text{mol/d}$, respectively ($P < 0.001$). The total tocopherol intake was the same for smokers and non-smokers.

Faecal total tocopherol excretion was significantly increased from control to intervention ($P = 0.046$) (Table 1). With respect to the subgroups, the total tocopherol excretion in smokers significantly increased from baseline and control to intervention ($P < 0.05$). At intervention, excretion significantly differed between smokers and non-smokers ($P = 0.008$).

For the whole study group, lipid-adjusted total tocopherol concentration in serum was significantly decreased at control compared with baseline and intervention period ($P < 0.01$). Similar relationships between the periods were present in non-smokers ($P < 0.05$) and in smokers, though in smokers

the decrease from baseline to control was a tendency only ($P = 0.057$).

Diphenols in stool

Although the analyses were carried out on lyophilised stool samples, the results were extrapolated to the daily excreted amount of fresh matter, because of the increased daily stool mass and DM (, unpublished results) at intervention (Table 2). The generation of the diphenols 2,3- and 2,5-DHBA in faeces was significantly increased from baseline to intervention ($P < 0.01$). The subgroup of non-smokers showed no changes regarding the diphenols, whereas in smokers concentrations during intervention increased compared with baseline and control ($P < 0.05$).

Blind tests (without faecal samples), tests with additional antioxidants (gallic acid, trolox; 0.04 M being the tenfold of the expected molarity of each diphenol) and tests without Fe and EDTA were also conducted. Diphenols were not detected in any of the incubation batches. The salicylic acid peak in the faecal chromatogram confirmed that salicylic acid was present in abundance. In contrast, catechol could not be detected. The correlation determined between total DHBA (based on 100 mg fresh matter) and 8-oxodG was $r = 0.328$ during PI ($P = 0.051$), $r = 0.354$ during PII ($P = 0.034$) and $r = 0.324$ during PIII ($P = 0.054$). Further, total DHBA (extrapolated to daily excreted fresh matter) and faecal Fe excretion were highly correlated ($r = 0.707$ for PI ($P < 0.001$), $r = 0.568$ for PII ($P < 0.001$) and $r = 0.744$ for PIII ($P < 0.001$)). Total DHBA (extrapolated to daily excreted fresh matter) and faecal water genotoxicity were negatively associated ($r = -0.273$ for PI ($P = 0.107$), $r = -0.313$ for PII ($P = 0.063$) and $r = -0.134$ for PIII ($P = 0.434$)).

Iron parameters

Fe intake increased steadily from baseline to control and again to intervention ($P < 0.001$) (Table 3). Similarly, Fe excretion increased with control and test bread v. baseline ($P < 0.01$). However, serum ferritin concentration was significantly lower at intervention than at baseline ($P < 0.05$). Excluding

Table 1. Changes in serum and stool total tocopherol concentrations after control bread consumption and intervention with blackcurrant seed press residue-enriched test bread considering smoking habits (Mean values and standard deviations)

	Baseline		Control bread		Test bread	
	Mean	SD	Mean	SD	Mean	SD
Serum ($\mu\text{mol}/\text{mmol}$ serum lipids)†						
All (n 36)	5.43 ^a	1.39	4.34 ^b	1.79	5.62 ^a	1.23
Smokers (n 16)	5.11 ^{a,b}	1.35	3.87 ^a	1.76	5.54 ^b	1.32
Non-smokers (n 20)	5.69 ^a	1.40	4.72 ^b	1.77	5.68 ^a	1.18
Stool ($\mu\text{mol}/\text{d}$)						
All (n 36)‡	23.7 ^a	14.9	26.2 ^{a,b}	19.7	28.1 ^b	17.8
Smokers (n 16)	27.5 ^a	18.6	26.9 ^a	17.4	36.9 ^{b*}	20.3
Non-smokers (n 20)	20.6	10.7	25.7	21.8	21.1	11.8

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P \leq 0.05$).

* Mean value was significantly different from that of the non-smokers ($P \leq 0.05$).

† Tocopherols were adjusted to serum lipids (TAG + cholesterol).

‡ Values were not distributed normally; significance was calculated by means of the Wilcoxon test.

Table 2. Concentration (mmol/d fresh matter) of 2,3- and 2,5-dihydroxy benzoic acid (DHBA) in stool, generated from salicylic acid *in vitro* after control bread consumption and intervention with blackcurrant seed press residue-enriched test bread considering smoking habits (Mean values and standard deviations)

	Baseline		Control bread		Test bread	
	Mean	SD	Mean	SD	Mean	SD
All (n 36)						
2,3-DHBA†	199 ^{a*}	131	220 ^{a,b}	130	271 ^{b*}	197
2,5-DHBA	107 ^a	74	125 ^{a,b}	82	156 ^b	113
Total DHBA	306 ^a	201	344 ^{a,b}	211	426 ^{b*}	308
Smokers (n 16)						
2,3-DHBA	229 ^a	163	236 ^{a,c}	91	373 ^b	246
2,5-DHBA	123 ^a	85	132 ^{a,c}	51	213 ^b	136
Total DHBA	351 ^a	242	368 ^a	139	586 ^b	379
Non-smokers (n 20)						
2,3-DHBA	175	96	206	155	189	89
2,5-DHBA	94	63	120	102	110	63
Total DHBA	270	157	324	257	299	151
P between smoking habits						
2,3-DHBA	NS		NS		0.010	
2,5-DHBA	NS		NS		0.007	
Total DHBA	NS		NS		0.011	

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P \leq 0.05$).

* Mean value was marginally significantly different from that for the control bread ($P \leq 0.1$).

† Values were not distributed normally; significance was calculated by means of the Wilcoxon test.

anaemic participants, ferritin concentrations were 27.0 (SD 17.2) $\mu\text{g/l}$ (PI, n 31), 27.8 (SD 16.1) $\mu\text{g/l}$ (PII, n 26) and 22.2 (SD 11.6) $\mu\text{g/l}$ (PIII, n 30) with $P < 0.05$ for both, baseline and control compared with intervention (Wilcoxon test).

Cyto- and genotoxicity of faecal water

To evaluate genotoxicity, HT29 clone cell viability was assessed before and after incubation with faecal water. Viability at intervention was significantly reduced compared with baseline and control ($P < 0.05$) (Table 4). In non-smokers, cell viability was significantly decreased from baseline to intervention ($P < 0.001$). There were no changes for smokers and non-smokers between the control and intervention

periods. After cell incubation with faecal water, tail intensity significantly increased during intervention compared with both baseline and control ($P < 0.05$). In smokers, no changes occurred throughout the study periods. In non-smokers, the tail intensity increased with intervention v. baseline period ($P < 0.05$). Cell viability and genotoxicity of faecal water correlated negatively at the control and intervention periods (PI: $r = 0.208$ ($P = 0.228$); PII: $r = 0.764$ ($P < 0.001$); PIII: $r = 0.301$ ($P = 0.075$)).

Urinary 8-oxo-2'-deoxyguanosine

The daily urinary 8-oxodG excretion was not affected by the control bread compared with baseline, both in the whole study population and in the subgroups of smokers and non-smokers (Table 4). There was a tendency in the daily excretion towards an increase for the whole study population after test bread compared with control ($P < 0.1$) and it was significantly increased in non-smokers ($P < 0.05$). There were no significant changes between the periods within the subgroups of users and non-users of hormonal contraceptives, although the 8-oxodG excretion at intervention was significantly higher in the non-users (1.21 nmol/kg per 24 h) compared with the users (0.76 nmol/kg per 24 h) ($P = 0.004$).

F₂-isoprostanes and prostaglandin F_{2 α} metabolite

Urinary 8-iso-PGF_{2 α} and 15-keto-dihydro-PGF_{2 α} were only analysed in samples collected from the control and intervention periods to follow both oxidative stress and the inflammatory response (Table 5). No significant changes were found for the whole study population, and for the smokers and non-smokers separated. Interestingly, the excretion of urinary 8-iso-PGF_{2 α} in volunteers using no hormonal contraceptive was significantly increased by the intervention.

Discussion

The consumption of bread enriched with blackcurrant PR produced effects on biomarkers related to oxidative stress via different PR components, for example, phytic acid, PUFA or Fe. The decreased serum total tocopherol achieved with control bread compared with baseline was possibly a result of replacing the

Table 3. Iron intake, iron excretion, and iron serum parameters after control bread consumption and intervention with blackcurrant seed press residue-enriched test bread (Mean values and standard deviations for thirty-six subjects)

	Baseline		Control bread		Test bread	
	Mean	SD	Mean	SD	Mean	SD
Intake (mg/d)†	10.4 ^a	1.1	12.0 ^b	1.4	12.8 ^c	1.5
Faecal excretion (mg/d)	6.37 ^a	3.60	8.24 ^b	5.08	9.52 ^b	5.14
Fe in serum (mg/l)	1.09	0.41	1.17	0.45	1.07	0.45
Ferritin ($\mu\text{g/l}$)†	23.9 ^a	17.7	21.6 ^{a,b}	17.0	19.2 ^b	12.7
Transferrin (g/l)	3.08 [*]	0.65	3.20	0.64	3.09 [*]	0.63
Transferrin saturation (%)	26.4	11.4	27.3	11.4	25.8	11.6

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

* Mean value was marginally significantly different from that for the control bread ($P \leq 0.1$).

† Intake achieved using PRODI[®] 5.4 software (Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, Germany).

Table 4. Cyto- and genotoxicity of faecal water (comet assay, given in fluorescence tail intensity) and 8-oxo-2'-deoxyguanosine (8-oxodG) in urine after control bread consumption and intervention with blackcurrant seed press residue-enriched test bread considering smoking habits (Mean values and standard deviations)

	Baseline		Control bread		Test bread	
	Mean	SD	Mean	SD	Mean	SD
Cell viability (%)						
All (<i>n</i> 36)†	98.8 ^{a*}	1.3	95.1 ^a	15.8	92.5 ^b	17.0
Smokers (<i>n</i> 16)	98.8	1.2	97.8	3.1	90.1	23.0
Non-smokers (<i>n</i> 20)*	98.7 ^a	1.4	92.9 ^{a,b}	21.0	94.5 ^b	10.3
Comet assay (%)						
All (<i>n</i> 36)	13.5 ^a	8.4	13.9 ^a	9.9	17.2 ^b	13.4
Smokers (<i>n</i> 16)	14.2	9.0	12.9	7.4	15.0	9.1
Non-smokers (<i>n</i> 20)	13.0 ^a	8.2	14.7 ^{a,b}	11.7	19.1 ^{b*}	16.1
8-OxodG (nmol/kg per 24 h)						
All (<i>n</i> 36)†	0.749	0.684	0.723	0.823	0.935*	1.236
Smokers (<i>n</i> 16)	0.805	0.845	0.693	0.829	0.889	1.624
Non-smokers (<i>n</i> 20)	0.705 ^{a,b}	0.541	0.747 ^a	0.838	0.971 ^b	0.853

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P \leq 0.05$).

* Mean value was marginally significantly different from that for the control bread ($P \leq 0.1$).

† Values were not distributed normally; significance was calculated by means of the Wilcoxon test.

normally consumed foods in favour of the control bread. Though tocopherol intake was increased with the control bread, suggesting an increase of serum tocopherol, single isomers may have been less absorbable, resulting in a decrease. This effect was reduced by the PR intake at intervention and serum total tocopherol concentration increased compared with the control bread. Serum total tocopherol concentrations showed no differences between smokers and non-smokers at any period, confirming the findings of Kontush *et al.* (7).

Blackcurrant berries and seeds contain significant quantities of proanthocyanidins (30,31). Because dietary polyphenols are virtually all degraded to monomers by normal gut bacteria, the phenol concentration in faecal water is high enough to bring about physiological changes in the stool (32). Due to low absorption rates of polyphenols, effects in the gastrointestinal tract might be even higher than in the body itself. Cyanidin-3-glycoside, a chief component of blackcurrant anthocyanins, was protective towards H₂O₂-induced DNA strand breaks in colonocytes *in vitro*, but failed at physiological dose rates in rats *in vivo* (31,33). Further, phytic acid from bread and also from PR might improve the antioxidant properties of both the breads.

Contrary to results achieved from studies on polyphenols and phytic acid, the combination of bread and PR increased the formation of diphenols compared with control bread, implying a decreased capacity in the scavenging of hydroxyl radicals with the consumption of the PR bread. Generation of the diphenols 2,3- and 2,5-DHBA remained unaffected by the bread itself. Smoking increased the generation of both diphenols at intervention compared with control and baseline. Thus, the faeces from smokers showed a reduced antioxidant capacity when PR bread was consumed. In addition to the polyphenols, the consumption of tocopherols should also point to an increase in the faecal antioxidant capacity rather than a decrease. However, it has been reported that tocopherols do not effectively scavenge the ubiquitously occurring hydroxyl radicals (34).

An analysis of the components of blackcurrant PR exhibited the presence of significant amounts of Fe in the PR (1). Therefore, the Fe parameters were also taken into consideration in this human intervention study. Most of the PR Fe probably came from the mill during crushing. Steel dust is a catalyst for Fenton-like oxidations (35). The increased Fe intake was not reflected in an improved Fe status of the

Table 5. 8-Iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} excretion (nmol/24 h) in urine after control bread consumption and intervention with blackcurrant seed press residue-enriched test bread considering smoking habits and the use of hormonal contraceptives (Mean values and standard deviations)

	8-Iso-PGF _{2α}					15-Keto-dihydro-PGF _{2α}				
	Control bread		Test bread		P*	Control bread		Test bread		P*
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
All (<i>n</i> 36)	9.32	3.16	9.71	2.99	0.495	2.30	0.89	2.47	0.91	0.176
Smokers (<i>n</i> 16)	9.41	2.41	9.71	3.31	0.590	2.38	1.10	2.33	1.00	0.428
Non-smokers (<i>n</i> 20)	8.56	2.16	9.28	2.54	0.323	2.15	0.60	2.57	0.89	0.409
Contraceptive users (<i>n</i> 22)	9.22	3.68	8.73†	2.68	0.518	2.16	0.70	2.22†	0.78	0.673
Non-contraceptive users (<i>n</i> 14)	9.48	2.22	11.26	2.87	0.042	2.53	1.13	2.87	0.98	0.146

* Between control and test bread for each prostate.

† Mean value was significantly different from that for the non-users of hormonal contraceptives ($P < 0.05$).

participants. In fact, most of the Fe remained in the intestinal lumen, able to act as a damaging factor⁽³⁶⁾. The concentration of the Fe-storage protein ferritin decreased with consumption of both the control and test bread, indicating an Fe deficiency in the body. An impairment of Fe status has also been described in women consuming wheat bread⁽³⁷⁾. The phytic acid in the grain potentially binds faecal Fe and decreases absorption. The ferritin decrease became even more pronounced with the test bread when excluding the anaemic participants, possibly because PR additionally contained phytic acid. In a prior study with a similar study design, 10 or 20 g PR were eaten daily in yoghurt for 4 consecutive weeks (, unpublished results). Here, ferritin concentrations also decreased significantly from baseline (pure yoghurt) to both intervention periods, suggesting that ferritin decrease is most probably due to the PR.

Phytic acid prevents the generation of hydroxyl radical by chelating Fe⁽³⁸⁾. Nevertheless, it appears questionable whether phytic acid has an impact on antioxidant capacity in the *in vitro* test system. First, phytic acid reduces the hydroxyl radical formation by trapping the Fe, but does not scavenge the hydroxyl radicals produced in the test system. However, phytic acid can have an impact on antioxidant conditions *in vivo* due to the absence of EDTA⁽³⁹⁾. Second, when EDTA, which is a stronger Fe scavenger than phytic acid, is added to the test system for *in vitro* aromatic hydroxylation, the Fe gets detracted from the phytic acid⁽¹⁴⁾. Contrary to phytic acid, EDTA is a promoter of hydroxyl radical generation because of its ability to form a free Fe coordination site, thus contributing to the oxidation processes^(38,40). It is generally assumed that radicals in stool and oxidation of membrane lipids and its chain-reaction products lead to *in vivo* DNA damage. The correlations determined between DHBA and 8-oxodG and faecal Fe excretion confirm this assumption. The high Fe content of the digest possibly increased the formation of hydroxyl radicals *in vivo*, exhausting faecal antioxidants, leading to a reduced antioxidant capacity of the stool. A study using dimethylsulfoxide to scavenge faecal hydroxyl radicals reported that a diet rich in fat and meat and low in fibre showed a thirteen times increased hydroxyl radical production than with a low-fat, vegetarian high-fibre diet⁽⁴¹⁾. In fact, there resulted a 42 % increased faecal Fe concentration in the high-meat diet. The concentration of faecal Fe is of no consequence in the test system utilising aromatic hydroxylation, since Fe is added in abundance to provoke the radical generation. However, faecal Fe might have exhausted faecal antioxidants in the body already.

It was shown that Fe^{2+/3+} also accounts for DNA damage⁽⁴²⁾. In the present study, faecal Fe concentrations remained unchanged between the control and test breads, but faecal water genotoxicity increased not with the control bread, but only with the test bread. Further, faecal water genotoxicity and faecal Fe concentration did not correlate at test bread consumption, suggesting that increased genotoxicity at intervention was caused by something other than the Fe.

In the present study, faecal water genotoxicity and generated DHBA were negatively associated. Consequently, if both DHBA and faecal water genotoxicity were increased by the intervention, the causes might be attributed to different factors in each case. Further, it has been under discussion that the intake of PUFA promotes DNA damage in the

body⁽⁴³⁾. This is a relevant factor since PR contains high amounts of these fatty acids (79 % PUFA of total fatty acid methyl esters⁽¹⁾) and 4 g PUFA were ingested daily with the test bread. Heat applied during seed processing and bread baking may have induced lipid peroxidation leading to the increased oxidative DNA damage by the faecal water at PR consumption. No correlations could be found between the genotoxicity of faecal water and the total tocopherol concentration of faeces. Testing on the cell viability supports the results of the comet assay. Cell viability decreased after incubating cells with faecal water from test bread consumption and both parameters correlated negatively at the control and intervention periods. Bile acids, not analysed here, are other possible contributors to elevated reactive oxygen species generation and DNA hydroxylation⁽⁴⁴⁾. In addition, fibre has a bile acid-binding capacity⁽⁴⁵⁾. Consequently, a change in bile acid concentration and pattern at control and intervention is probably due to increased daily fibre intakes compared with baseline (31 % bread, 54 % bread + PR).

Correlation values of serum total tocopherol or total tocopherol intake in the present study confirm that dietary consumption of antioxidants, particularly of vitamin E, is associated with little or no effects on the urine 8-oxodG concentration^(21,33,46). Gackowski *et al.* described hydroxyl radicals as being the major source of 8-oxodG formation⁽⁴⁷⁾. To attack DNA, these radicals need to be in its immediate vicinity. However, lipophilic vitamins are not usually located near DNA molecules. Non-smokers excreted significantly more 8-oxodG during PR consumption than during intake of control bread. Noticeably, smokers had a two-fold higher standard deviation than non-smokers. Curiously, smokers showed particularly individual reactions to the substances in PR. Generally, 8-oxodG excretion increased with PR bread, although the extent of the increase was influenced by different factors such as smoking habits, the use of hormonal contraceptives, and other, unidentified parameters. There was no correlation between 8-oxodG excretion and faecal water genotoxicity. These results support another study that showed no relationship between faecal water genotoxicity and lymphocyte DNA damage using the comet assay⁽¹⁶⁾. An Fe-dependent increase of 8-oxodG in lymphocytes was found with acute Fe load⁽⁴⁸⁾. In the present study, none of the measured serum or faecal Fe parameters was clearly associated with the urinary 8-oxodG excretion. Thus, the PR component responsible for the increase of urinary 8-oxodG concentration could not be identified.

No differences were shown for prostane excretion in the total study population. However, different outcomes were apparent when looking at the individual subgroups. While hormonal contraceptives obviously protected a PR-induced non-enzymic lipid peroxidation, smoking, contrary to several reports, caused no significant impact⁽⁴⁹⁾. There was no association found between prostane excretions and other tested parameters. England *et al.* found an association between plasma isoprostane 8-epi-PGF_{2α} and lymphocyte 8-oxodG⁽⁵⁰⁾. In the present study, only when urinary 8-oxodG and prostanes were analysed in the defined subgroups, strong correlations appeared with PR consumption between 8-oxodG and the oxidative stress marker 8-iso-PGF_{2α} excretion for non-smokers and non-users of hormonal contraceptives. Noticeably, these are the subgroups not influenced by special oxidants or antioxidants, in contrast to smokers

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(increased reactive oxygen species) or users of hormonal contraceptives (antioxidant protection). Further, it was shown that a PUFA-rich diet may increase plasma $\text{PGF}_{2\alpha}$ concentration⁽⁵¹⁾. Thus, the 8-iso- $\text{PGF}_{2\alpha}$ increase might be attributed to the increased PUFA intake with the PR bread.

Conclusion

Testing complex food matrices makes it difficult to arrive at clear and distinct outcomes compared with single intervention substances. Consequently, effects due to an intervention with berry PR cannot be accredited to one single substance. Nevertheless, the following statements can be presumed: PR consumption resulted in increased faecal Fe concentrations, decreased faecal antioxidant capacity and increased urine 8-oxodG excretion. The increased genotoxicity of faecal water and partially increased prostane excretion could be due to the higher PUFA intake. PR phytic acid is assumed to account for the decrease in serum ferritin. Furthermore, factors associated with lifestyle, such as smoking habits or the use of hormonal contraceptives, have an impact on the correlations described above. In summary, consuming black-currant PR-enriched bread for 4 weeks has adverse effects on the antioxidant status in the body, as serum and stool total tocopherol concentrations were increased. The antioxidant properties of tocopherols had no effect on the measured biomarkers associated with oxidative stress.

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MANUSCRIPT III (SUBMITTED)

Tocopherol isomer pattern in serum and stool of human following consumption
of black currant seed press residue administered in whole grain bread

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Title: Tocopherol isomer pattern in serum and stool of human following consumption of black currant seed press residue administered in whole grain bread

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Short title: Black currant seed press residue consumption

Abbreviations: PR: Press residue; CEHC: carboxyethyl-hydroxychromanol; NDF: neutral detergent fibre; ADF: acid detergent fibre; TEAC: Trolox equivalent antioxidant capacity; BMI: body mass index

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Abstract

Background & aims: Serum γ -tocopherol is thought to be associated with human health. The dietary influence of tocopherol and fibre-rich black currant seed press residue on serum and stool tocopherol concentration was investigated in a controlled human intervention study.

Methods: 36 women consumed bread enriched with black currant press residue (4 weeks). The resultant faecal and serum tocopherol concentrations were compared with those after a period consuming control bread without press residue and a normal baseline diet. Fibre intake and excretion, antioxidant capacity (TEAC), and vitamin C concentrations in serum and urine were also determined. Samples were obtained with a 5-day standardised diet at the end of each period. **Results:** The press residue bread lead to significantly increased β -, γ -, δ - and total tocopherol intake, serum α -, β -, γ - and total tocopherol concentration (with and without lipid adjustment), fibre intake and urinary vitamin C concentration compared to control bread ($P<0.05$). Faecal excretion of total tocopherols and fibre increased compared to baseline ($P<0.05$). **Conclusions:** Fibre intake and excretion influence total tocopherol concentration in lipid-adjusted serum and in stool. The outstandingly high increase of serum γ -tocopherol concentration through seed press residue consumption could be due to a presumed interruption of the enzymatic tocopherol degradation mechanism by bread constituents.

ClinicalTrials.gov ID: NCT00662766

Keywords: tocopherol isomer, serum, stool, fibre, black currant

Introduction

Vitamin E and in particular γ -tocopherol have antioxidant properties believed to be associated with the development of atherogenesis, coronary heart diseases and cancer.¹⁻³

As for all fat-soluble substances, the presence of lipids is essential for vitamin E absorption.⁴

Moreover, pancreatic enzymes are necessary for the saponification of esterified tocopherols in order for them to be incorporated into chylomicrons which essentially contain bile acids and apolipoprotein B.^{5,6} Nevertheless, appreciable tocopherol concentrations remain in the gut. The above described mechanism can be impeded by nutrients such as fibre or by bile acids.^{5,7}

Vitamin E comprises eight isomers (α -, β -, γ -, δ -tocopherol and α -, β -, γ -, δ -tocotrienol). After partial absorption in the small intestine, α -tocopherol is predominantly incorporated into plasma lipoproteins *via* the α -tocopherol transport protein in the liver, while β -, γ -, and δ -tocopherols are basically metabolised and excreted as carboxyethyl-hydroxychromanols (CEHC).^{8,9} Therefore, α -tocopherol constituting >93% of total serum tocopherol represents the major serum tocopherol. There is no interference between α - and γ -tocopherol at the enterocyte absorption site and the discrimination of both tocopherols is a post-absorptive process.¹⁰ Only small quantities of the α -tocopherol are metabolised by enzymes in the body and its disappearance results from other mechanisms, e.g. excretion *via* bile.¹¹ Excessive administration of α -tocopherol decreases serum γ -tocopherol concentration by allegedly increasing the γ -tocopherol metabolism.¹²⁻¹⁴ However, other studies describe a subsequent accumulation of γ -tocopherol when tocopherols are applied in combination with substances such as lignans that interrupt tocopherol side-chain degradation.¹⁵⁻¹⁷

In the present study it was tested, whether consumption of tocopherol-containing black currant seed press residue (PR) altered the serum tocopherol concentration and isomer pattern and if it had effects on parameters of the antioxidant status. Moreover, it was considered, whether fibre intake and excretion had an influence on tocopherol concentration in serum and stool.

Materials and methods

Test substance

Black currant seeds were pressed and subsequently ground for purposes of edibility. The obtained PR had 48.2 g/100 g total fibre, 25.7 g/100 g fat (79% polyunsaturated fatty acids), and 22.5 g/100 g of crude protein.¹⁸ PR contained α -tocopherol (2.82 mg/100 g PR),

γ -tocopherol (5.97 mg/100 g PR), and also 0.12 mg γ -tocotrienol/100 g PR. The PR was baked into bread consisting of sour dough (19%), wheat flour (24%), rye flour (7%), and crushed rye grain (8%) and a small quantity of oat (3%) and flax seeds (3%). To the test bread 8 % (w) black currant PR were added. Total fibre was 9.0 g/100 g for the control bread and 11.0 g/100 g for the test bread. Gallic acid equivalent (total phenols) in control bread was 55.4 mg/100 g and 69.1 mg/100 g in test bread. Using a hydrophilic solvent, the TEAC value was measured as 3.45 μ mol/g and 4.67 μ mol/g, while the vitamin C concentration was 3.76 mg/100 g and 4.97 mg/100 g in control and test bread, respectively. The tocopherol concentration of 100 g control and test bread was 0.319 and 0.547 mg α -tocopherol, 0.436 and 0.413 mg β -tocopherol, 0.773 and 1.573 mg γ -tocopherol, and 0.087 and 0.115 mg δ -tocopherol, respectively.

Study design, population, food intake and sample preparation

In order to test the PR-enriched bread, initial baseline samples (PI) were taken during a standardised diet from 36 females (16 smokers, 20 non-smokers) (FIGURE 1). Further, 250 g/d (fresh weight) of control bread (PII) and the same amount of test bread which lead to an intake of 20 g PR/d (PIII) were consumed, each for a period of four weeks. During the last five days of each period, a standardised diet was administered again, while the bread consumption was continued. Participants were not told whether control or test bread being consumed but test bread was visually identifiable. The standardised diet comprising of a warm meal, fruits and vegetables, bread, bread-spreads and beverages, was weighed and prepacked for each day. All uneaten portions were returned for quantification of the actual food consumption. The intake of energy, fat, protein, and carbohydrates was verified using the Prodi[®] 5.4 software (Nutri-Science GmbH, Freiburg, Germany). For intake of fibre and tocopherols, the respective concentrations in the provided foods were analysed instead of using the Prodi[®] software.

During the last three days of each period, stool and urine were collected quantitatively, and a blood sample was taken. Aliquots of the 24-h urine samples over the three days were combined to one sample and stored at -20 °C. The weight and pH-value of the 3-day stool output were measured. The samples were frozen until the study ended. Then, the 3-day total stool collections were defrosted, mixed, lyophilised, ground and further stored at -20 °C. Dry matter was measured in aliquots of the 3-day stool samples by recording weight loss after drying for 24 h at 105 °C. Blood samples were centrifuged. The obtained serum was stored at -80 °C until analysed.

Participants mean age was 24 ± 3 (18 - 33) years, the mean height was 170 ± 7 (157 - 186) cm, and the mean weight was 67 ± 11 (53 - 97) kg resulting in a mean BMI of 23.3 ± 3.7 (17.9 - 32.8) kg/m². All test persons gave their written consent after they had been informed about the purpose, the course and their duties for the study. The study was approved by the Ethics Committee of the Friedrich Schiller University of Jena, Germany (Approval Number: 1485-01/05). Exclusion criteria from the study were going on a diet during the study, participation in intensive exercise, diagnosed diseases interfering with the study, and the use of nutritional supplements. With an initial number of 40 volunteers for the study, four participants dropped out of the study for individual reasons (e.g. illness or lack of time).

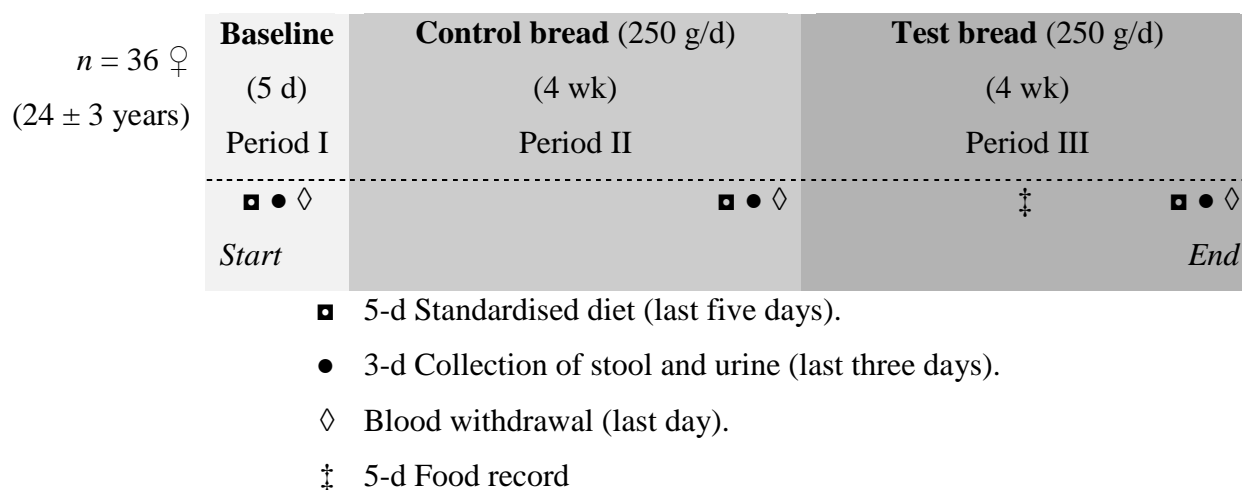


FIGURE 1 Study design for testing black currant seed press residue (PR) baked into whole grain bread.

Test bread included 8% black currant PR but was otherwise like the control bread. Baseline was defined by a normal but standardised diet. The same standardised diet was administered at the last five days of Period II and III together with the control and test bread consumption.

TEAC

For the determination of the Trolox equivalent antioxidant capacity (TEAC) in serum and urine, sample aliquots were diluted in distilled water.¹⁹ ABTS (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt) was reduced to a radical using K₂O₈S₈ and added to the sample. After 1 min, photometric analysis at $\lambda=734$ nm was achieved using blank value (distilled water) and Trolox[®] (Sigma-Aldrich, Taufkirchen, Germany) standard solutions for quantification. The coefficient of variation of TEAC value obtained

from four measurements on two different days including the lowest and highest calibration levels was 5.6%.

Vitamin C

The method according to Schlesier et al.¹⁹ was used to measure vitamin C concentrations by diluting an aliquot of the urine or serum sample with trichloroacetic acid followed by centrifugation. The supernatant fraction and dinitrophenylhydrazine reagent including copper was incubated to catalyse the reduction of ascorbic acid to dehydroascorbic acid. Sulphuric acid was added before the photometric analysis at $\lambda=520$ nm was performed. A calibration curve of ascorbic acid corrected with blank values (water) was used for quantification (Fluka, Buchs, Switzerland). The coefficient of variation of vitamin C obtained from six analyses per day including the lowest and highest calibration levels was 5.3%.

Tocopherols

The serum aliquot was added to an ethanol BHT solution, mixed, and extracted with *n*-hexane-BHT solution. The tocopherol concentration of extract was measured using HPLC/fluorescence.²⁰

Each aliquot of food sample from the standardised diet, control and test bread as well as lyophilised stool was mixed with ascorbic acid and aqua distillata and incubated with potassium hydroxide solution for 30 min at 80 °C.²⁰ The samples were extracted with *n*-hexane-BHT solution and measured using HPLC/fluorescence. For every batch run, a standardised milk powder (BCR-421, Promochem, Wesel, Germany) was processed analogically. For purposes of tocopherol calibration, standards from Calbiochem, Darmstadt, Germany (Isomer kit no. 613424) were used. Total tocopherol was calculated as sum of the measured isomers. The coefficients of variation of the tocopherol isomers obtained from four analyses per day including the lowest and highest calibration levels were 4.0% (α), 2.4% (β), 3.1% (γ) and 2.9% (δ).

Total fibre

The total fibre concentration in the breads, in the food from the standardised diet and in the stool was determined using BIOQUANT[®] Total Dietary Fibre (Merck, Darmstadt, Germany) employing the enzymes α -amylase, protease, and amyloglucosidase (AOAC International, 1995). In the FIBERTEC E[®], the sample was filtered and washed with ethanol, acetone and

petroleum ether. The remaining sample was reweighed as fibre. In parallel samples, the weight of retained protein and ashes was determined and subtracted from the remainder.

Statistical analysis

Normal distribution of results was determined by means of the Kolmogorov-Smirnov test using the SPSS 14.0 software package (SPSS Inc., Chicago, IL, USA). Unless specified elsewhere, significance was tested using repeated measure analysis of variance. The one-sided Mann-Whitney U test was used to compare smokers and non-smokers. For correlation analysis, the Pearson's correlation coefficient was determined. Values were referred to as significant at $P < 0.05$.

Results

Food intake during the standardised diet

The administered standardised diet met the dietary guidelines of the German Nutrition Society.²¹ Despite the use of 5-day standardised diets for sample taking, there were differences in the actual food intake between these 5-day periods as participants were free to consume either all or some of the supplied diet. Nevertheless, fat intake remained stable throughout the periods (TABLE 1). Energy and fibre intake were increased and vitamin C intake was reduced during both the control and test bread periods when compared to the baseline period. The increased energy intake was due to increased carbohydrate consumption. Test bread consumption led to less protein, carbohydrate and vitamin C intake and more fibre intake when compared to control bread consumption.

α -Tocopherol intake was similar for all test periods (TABLE 1). Intake of β -, γ -, and δ -tocopherol was higher during control and test bread consumption than at baseline ($P < 0.001$). Comparing control and test bread, the intake of β -tocopherol was lower with test bread ($P < 0.01$) whereas that of γ - and δ -tocopherol was higher ($P < 0.001$). Total tocopherol intake was highest with test bread consumption ($51.4 \pm 8.2 \mu\text{mol/d}$) and lowest at baseline ($42.0 \pm 5.9 \mu\text{mol/d}$) and was between these two values for control bread consumption ($48.3 \pm 6.7 \mu\text{mol/d}$; $P < 0.01$ between all periods).

TABLE 1

Food intake¹ and parameters of the faecal matrix during a 5-day standardised diet
with 3-day stool collection following consumption of black currant seed press
residue in bread (means \pm SD)

Intake/parameters		Baseline	Control bread	Test bread
Energy intake [MJ/d]		7.35 \pm 8.11 ^a	8.00 \pm 1.06 ^b	7.77 \pm 1.19 ^b
Fat intake [g/d]		75.3 \pm 11.1	74.2 \pm 15.3	74.4 \pm 16.3
Protein intake [g/d]		68.3 \pm 7.5 ^a	70.7 \pm 10.8 ^b	66.7 \pm 12.3 ^a
Carbohydrate Intake [g/d]		199 \pm 23 ^a	237 \pm 25 ^b	228 \pm 29 ^c
Fibre intake [g/d]		34.4 \pm 4.3 ^a	47.5 \pm 5.9 ^b	49.4 \pm 6.3 ^c
Vitamin C intake [mg/d]		148 \pm 30 ^a	134 \pm 32 ^b	117 \pm 35 ^c
Tocopherol intake [μ mol/d]	α	26.2 \pm 3.5	26.1 \pm 4.0	25.4 \pm 4.9
	β	5.49 \pm 0.85 ^a	7.54 \pm 1.1 ^b	7.00 \pm 1.09 ^c
	γ	9.71 \pm 1.59 ^a	13.5 \pm 1.7 ^b	17.7 \pm 2.3 ^c
	δ	0.65 \pm 0.10 ^a	1.15 \pm 0.12 ^b	1.28 \pm 0.15 ^c
Fibre excretion	[g/d]	8.8 \pm 4.0 ^a	11.5 \pm 6.2 ^b	13.5 \pm 8.2 ^b
	[g/100 g fresh stool]	8.3 \pm 2.0 ^a	9.6 \pm 2.3 ^b	10.5 \pm 2.7 ^c
Tocopherol excretion [μ mol/d] ²	α	16.1 \pm 10.2	15.1 \pm 11.4	15.8 \pm 10.5
	β	4.12 \pm 2.59 ^a	5.34 \pm 3.74 ^b	5.04 \pm 3.01 ^b
	γ	2.87 \pm 1.90 ^a	4.62 \pm 3.56 ^b	5.96 \pm 3.69 ^c
	δ	0.61 \pm 0.77 ^a	1.14 \pm 1.65 ^b	1.36 \pm 1.68 ^b
Stool mass [g/d]		112 \pm 63	123 \pm 71	125 \pm 53
Dry matter stool [%]		24.5 \pm 5.2 ^a	27.7 \pm 5.0 ^b	28.6 \pm 4.2 ^b
pH value stool		6.66 \pm 0.34	6.67 \pm 0.33	6.58 \pm 0.37

^{a,b,c} Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

¹ Calculated using Prodi[®] 5.4 software; fibre intake was analysed at instituts laboratory.

² Values were not distributed normally, significance was calculated by means of the Wilcoxon test.

Parameters of the faecal matrix

While daily fibre and dry matter excretion in stool were increased with control and test bread ($P < 0.01$), there was no change in the daily faecal mass excretion or faecal pH-value throughout the study (TABLE 1).

In keeping with the unchanged α -tocopherol intake there were no changes in the α -tocopherol excretion between the periods (Table 1). Compared to baseline, β - and δ -tocopherol excretion were increased for control and test bread ($P < 0.05$). γ -Tocopherol excretion was lowest during baseline and highest during test bread consumption ($P < 0.05$). The total tocopherol excretion was $23.7 \pm 14.9 \mu\text{mol/d}$ (baseline), $26.2 \pm 19.7 \mu\text{mol/d}$ (control), and $28.1 \pm 17.8 \mu\text{mol/d}$ (test bread), respectively ($P < 0.05$ between baseline and test bread).

Serum tocopherols

Serum α -tocopherol concentration was similar for baseline and test bread consumption but was significantly lower for control bread ($P < 0.01$) (TABLE 2). Since α -tocopherol in serum constitutes the main tocopherol isomer, regarding the total tocopherol concentration there were comparable statistical relations between the periods (PI: $31.4 \pm 7.8 \mu\text{mol/L}$; PII: $26.6 \pm 11.9 \mu\text{mol/L}$; PIII: $32.1 \pm 6.6 \mu\text{mol/L}$; $P < 0.01$).

TABLE 2

Serum tocopherol concentration with and without lipid adjustment following consumption of black currant seed press residue in bread (means \pm SD)

Parameters		Baseline	Control bread	Test bread
Tocopherol [$\mu\text{mol/L}$]	α	29.6 ± 7.5^a	25.0 ± 11.4^b	30.1 ± 6.2^a
	β	0.57 ± 0.18^a	0.45 ± 0.16^b	0.54 ± 0.21^a
	γ	1.18 ± 0.34^a	1.17 ± 0.46^a	1.49 ± 0.58^b
Lipid-adjusted tocopherol [$\mu\text{mol/mmol}$ serum lipids]	α	5.13 ± 1.35^a	4.08 ± 1.73^b	5.26 ± 1.17^a
	β	0.10 ± 0.03^a	0.07 ± 0.02^b	0.09 ± 0.03^a
	γ	0.20 ± 0.05^a	0.19 ± 0.07^a	0.26 ± 0.10^b

^{a,b}Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

δ -Tocopherol was below detection limit

Lipid adjustment represents the relation of tocopherol to cholesterol + triacylglycerols.

Intervention with test bread increased the serum γ -tocopherol concentration compared to baseline and control ($P<0.001$). Moreover, β -tocopherol concentration was significantly lower for control bread consumption compared to the other periods ($P<0.05$).

Lipid-adjusted tocopherol concentrations revealed identical findings to those for free, unadjusted tocopherol concentrations in serum. The effect of PR consumption on serum tocopherol status was not modified by the BMI of the participants.

TEAC and vitamin C

Serum TEAC values remained stable between each period (TABLE 3). TEAC values in urine increased with control and test bread consumption compared to baseline ($P<0.001$), whereas vitamin C concentrations in urine were only slightly increased for test bread consumption ($P=0.059$). Vitamin C concentration in serum was significantly increased for control bread consumption compared to baseline ($P<0.05$).

TABLE 3

TEAC and vitamin C concentrations in serum and urine during the 5-day standardised diet with 3-day urine collection following consumption of black currant seed press residue in bread (means \pm SD)

Parameters	Baseline	Control bread	Test bread
TEAC serum [mmol/l]	2.85 \pm 0.24	2.87 \pm 0.26	2.82 \pm 0.32
TEAC urine [mmol/l]	2.51 \pm 1.06 ^a	3.16 \pm 1.27 ^b	3.13 \pm 1.12 ^b
Vitamin C serum [μ mol/l]	92 \pm 28 ^a	101 \pm 36 ^b	97 \pm 39 ^{a,b}
Vitamin C urine [μ mol/d]	328 \pm 169	316 \pm 172	364 \pm 236 ¹

^{a,b}Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

TEAC Trolox equivalent antioxidant capacity

¹ $P=0.059$ between control and test bread

Discussion

Wheat flour, especially whole grain, may considerably contribute to the general tocopherol supply.²² An increased consumption of bread led to the raised total tocopherol intake at control compared to baseline. The addition of PR to the bread further increased the total tocopherol intake. However, although bread and PR were sources for α -tocopherol in the respective periods, the intake of α -tocopherol was identical between all periods. This was probably because α -tocopherol-rich dishes consumed during the normal diet were substituted by the study breads. In contrast, β -, γ -, and δ -tocopherol intake drastically increased for control bread and further more for PR bread. There is a clear difference in the pattern of the consumed tocopherols from that in serum tocopherols (TABLE 1, 2). In serum, no δ -tocopherol was detected, and β -tocopherol and γ -tocopherol were present in low concentrations compared to α -tocopherol. Consuming PR bread, a noticeable increase of approximately 20% in β -tocopherol and 30% in γ -tocopherol was evident compared to consuming control bread. In comparison, in a study involving participants with similar characteristics to the present study wherein 5.4 mg γ -tocopherol were consumed daily for four weeks in addition to a normal diet (present study: at intervention only 1.75 mg/d more than at control diet), the serum γ -tocopherol concentration remained unchanged.²³ Thus, in the present study the increase of γ -tocopherol concentration in serum was disproportionately high. A foregoing study in analogical design to the present study consuming PR mixed into yoghurt has proved that the effect must be exclusively due to the combination of bread with PR. In that study, volunteers consumed the same amount of PR resulting in no increase in the serum γ -tocopherol concentration (baseline: 1.22 ± 0.37 mmol/l, 20 g/d PR consumption: 1.34 ± 0.42 mmol/l; $P=0.216$). A possible explanation for high γ -tocopherol concentrations and perhaps also for β -tocopherol concentrations, although not yet described in literature, could be the metabolic side effects related to the consumption of a phenolic, lipophilic substance known as alkylresorcinol found in wheat and rye husks.²⁴ Together with γ -tocopherol, the alkylresorcinol successfully competes for tocopherol- ω -hydroxylase, a degradation enzyme, leading to an accumulation of γ -tocopherol in serum. In rats consuming alkylresorcinol, γ -tocopherol concentration increased in the liver and lung, but not in serum.^{25,26} Increased plasma concentrations in rats were found by substances such as sesamin that is more active in blocking the side-chain β -oxidation.^{27,25} Also in human plasma γ -tocopherol concentration increased with sesamin intake¹³ and γ -CEHC excretion decreased.²⁵ Unfortunately, the γ -CEHC concentrations were not measured in the present study. The question is why the

effect was only present for PR bread but was not seen in the control bread compared to baseline? It can be speculated that this effect does not become obvious until the γ -tocopherol intake reaches a specific level.

In contrast, the serum concentration of α -tocopherol can be increased by other mechanisms, such as epicatechin or quercetin regenerating the α -tocopheroxyl radical.²⁸ The changes in serum γ -tocopherol concentrations found in the present study were presumably not caused by the PR anthocyanins since according to a study in rats fed an anthocyanin-rich black currant concentrate, the rats showed no changes in liver and plasma tocopherols.²⁹

Fibre intake was significantly influenced by the different breads consumed ($P < 0.05$ between all periods). The fibre intake for the control bread was 22.5 g/d and 27.5 g/d for the test bread. In comparison, a hypothetical intake of 250 g wheat brown bread yields 11.6 g fibre. Though the fibre intake and also excretion increased with control and test bread, daily total fibre intake and excretion did not correlate indicating that fibre processing in the gastrointestinal (GI) tract varies for each individual. This distinctiveness could be due to the different food intake of each participant during the four weeks of bread consumption because except for the 5-day standardised diet at the end of the control and test bread period, the subjects were free to choose the foods that they consumed. It has been established that different food types lead to alterations in the faecal microbiota and fermentation processes.^{30,31} Whereas the participants consumed 49.4 ± 6.3 g/d fibre during the 5-day standardised conditions of the test bread period, they consumed 37.6 ± 11.0 g/d fibre on the other days of test bread consumption calculated from a 5-day food record within the first three weeks of test bread consumption (FIGURE 1). The large standard deviations in the non-standardised diets demonstrate the actual individualism regarding food intake which might have inhibited the conclusive effects of PR intervention. The faecal fibre concentration correlates positively with the faecal tocopherol concentration ($P < 0.001$ for all isomers and periods). Most studies focus on tocopherol intake and mainly disregard faecal excretion whereby there is a lack in information concerning the association of tocopherols and fibre in the GI tract. Schaarmann et al.³² found no correlation between faecal tocopherols and fibre in their studies involving oat bran in women. Harris et al.³³ describe the binding ability of fibre as varying, with wheat bran showing good binding properties. Due to the flexible structure of the fibre surface, they are able to bind and enclose water molecules including the dissolved substances⁷. In this way, fibre may further partition hydrophobic solvents containing tocopherols and possibly delay their absorption. Riedl et al.³⁴ described that serum α -tocopherol concentration was unaffected by the parallel consumption of different fibre types together with α -tocopherol. In contrast, our results show a significant

association of lipid-adjusted serum total tocopherol concentration with PR bread consumption to fibre intake ($r=-0.570$, $P=0.0003$) and fibre excretion ($r=0.345$, $P=0.040$), though it is not clear why there is a difference in the proportions for intake and excretion.

Vitamin C is closely connected to the regeneration of tocopherol and was therefore measured in urine and serum.³⁵⁻³⁸ PR intervention showed no effect on serum vitamin C concentration and antioxidant capacity using TEAC assay, but vitamin C excretion in urine showed a tendency to increase. Through the increased serum tocopherol concentration there was possibly no need for the regeneration of the constantly generated tocopheroxyl radicals by vitamin C, which was then not exhausted. Saturated vitamin C serum levels again result in lowered tubular reabsorption explaining the elevated vitamin C excretion.³⁹ According to sources in the literature, the present serum vitamin C concentrations were in the range of this ceiling effect. Hence the increased antioxidant capacity using TEAC assay in urine could be due to the increased vitamin C concentration in urine.^{19,40} Uric acid remained unaffected throughout the study (results not shown), thus, the changes of the urine TEAC values cannot be attributed to this antioxidant.

The significance of the study is limited by the fact that as the participants were only women results cannot be applied to men due to differences in metabolism. Further, participants were in a narrow range of age and results cannot be generalised to younger or older people.

In conclusion, the combined consumption of whole grain bread and black currant PR leads to increased serum β - and γ -tocopherol concentrations. However, neither bread nor PR alone can cause this effect. Thus, the effect might be attributed to substances that operate in a similar manner to alkylresorcinol from wheat or rye grain. In parallel, antioxidant capacity in urine was most likely supported by urinary vitamin C during test bread consumption. Furthermore, increased fibre concentration in stool increased concentrations of all faecal tocopherol isomers independent of baseline, or consumption of control or test bread. In addition, fibre intake and faecal excretion have an influence on the serum lipid-adjusted total tocopherol concentration. The increased serum γ -tocopherol concentration can be considered to be beneficial due to its different properties to α -tocopherol. γ -Tocopherol can for example trap mutagenic electrophiles, and finally, a decreased serum γ -tocopherol concentration is associated with an increased risk of coronary heart disease.^{41,42} If the elucidated mechanism regarding the increase of serum γ -tocopherol can be verified in future studies, a fortification of γ -tocopherol in whole grain products should be considered.

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FINAL DISCUSSION

4 FINAL DISCUSSION

4.1 POTENTIALLY HEALTH-BENEFICIAL SUBSTANCES IN BLACK CURRANT BERRY SEED PRESS RESIDUES

Before human intervention studies could take place, analyses of the different press residues (PR) were carried out in order to define substances with promising health-beneficial attributes. The leading substances of different PR species vary and cannot be generalised for all the species (MANUSCRIPT I). They all contained moderate to high concentrations of tocopherols, had a favourable $n-6/n-3$ ratio and a high PUFA content of >65% with linoleic acid predominating (>34%). Black currant PR was analysed in more detail leading to the result that tocopherols and fibre were termed as its main ingredients (MANUSCRIPT I - TABLE 1). For safety reasons, microbiological analyses were conducted (TABLE 2). These revealed that the concentrations of yeasts, mould and bacteria measured were all below the upper levels considered safe for ground grain products according to the values released by the German Society of Hygiene and Microbiology (Regulation (EU) 2073/2005).

TABLE 2

Microbiological characteristics* of black currant seed press residue [colony forming units/g].

Total counts	Coliforms	<i>E. coli</i>	Entero- bacteriaceae	Yeasts/mould	Salmonella
2.6×10^3	3.3×10^3	3.0×10^{-1}	3.3×10^3	2.0×10^3	none

* Analyses were conducted by the laboratories of the Fraunhofer Institute for Process Engineering and Packaging IVV, Freising, Germany.

The inositol hexaphosphates of the black currant PR are of final importance for interpreting the results of HIS II (TABLE 3). Black currant PR has relatively high concentrations of phytic acid and its hydrolysed inositol phosphates. Depending on the batch of seeds used, concentrations of the summed inositol phosphates varied about sevenfold.

Summing up the inositol-phosphates of the breads, test bread contained considerably more inositol-phosphates than control bread. Ingredients for both breads were the same, but for the test bread a bigger piece of yeastless sponge dough had to be used. This is necessary for

dough fermentation. The yeastless sponge dough contains microorganisms owing the enzyme phytase which degrades phytic acid to lower phosphorylated forms IP₁-IP₅ (CORSETTI & SETTANNI, 2007). Thus, for the test bread more phytase was brought into the dough producing more hydrolysis products. By the yeast sponge additionally the pH-value can have lowered, which increases the phytase activity (LEENHARDT *et al.*, 2005).

TABLE 3

Concentrations of inositol hexaphosphate* (IP₆) and its intermediate inositol phosphates (IP₅, IP₄, IP₃) in black currant press residues (PR) and breads administered in intervention study II.

	IP ₆	IP ₅	IP ₄	IP ₃	Sum IP ₃ -IP ₆
	[μmol/g]	[μmol/g]	[μmol/g]	[μmol/g]	[mmol/100 g]
PR batch I	46.7	1.23	traces	n.d.	4.80
PR batch II	6.30	traces	n.d.	n.d.	0.63
Control bread	2.57	0.30	0.73	1.37	0.50
Test bread	1.57	0.77	4.47	2.07	0.89

n.d.: below detection limit; batch I used for human intervention study I; batch II used for human intervention study II

* Analyses were conducted by the laboratories of Ralf Greiner (Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany).

About 50% of the PR consisted of fibre (MANUSCRIPT I). Consequently, test bread with PR contained more fibre (11.0 g/100 g) than the control bread (9.0 g/100 g). By the intake of 20 g PR/d (HIS I) 9.6 g fibre are consumed. For a better characterisation of the fibre, a detergent fibre analysis was performed with black currant PR. The NDF was 42.5 g/100 g total fibre (insoluble fibre), the soluble fibre was 57.5 g/100 g total fibre. Thereby, ADF was representing 18.5 g/100 g total fibre (MANUSCRIPT I).

Seed press residues were generally high in tocopherols and tocotrienols, particularly PR of black currant and elder. Black currant batches highly differed in their concentrations. The batch used for HIS I for example contained about the threefold of α - or β -tocopherol and about the 1.4-fold of γ - and δ -tocopherol compared to PR for HIS II (MANUSCRIPT I – TABLE 1). Instead, the batch used for HIS I contained no tocotrienols at all and additionally the fat content was lower than in PR for HIS II. The concentration of α -tocopherol in PR used for HIS I was 8.94 mg/100 g PR, that for HIS II was 2.82 mg/100 g PR. For comparison,

chicken, rice, oat, apple, broccoli or carrot have α -tocopherol concentrations between 400-700 $\mu\text{g}/100\text{ g}$ edible portion, butter or egg, olive and safflower oil have concentrations below 50 $\mu\text{g}/100\text{ g}$ edible portion (EGGERMONT, 2006).

Anthocyanins are the dominating phenolic compounds in black currant berries (MÄÄTTÄ-RIIHINEN *et al.*, 2001). As seen for the inositol-phosphate and tocopherol contents, also the anthocyanin concentrations greatly vary between batches (TABLE 4). Nevertheless, black currant PR has appreciable concentrations of anthocyanins but when measured by edible portions the PR is far behind those concentrations found in black currant berries or juices. Depending on the cultivar, berry anthocyanin concentrations were between 587 and 322 $\text{mg}/100\text{ g}$ fresh weight and were 59 $\text{mg}/100\text{ g}$ in black currant juice (WU *et al.*, 2004; NIELSEN *et al.*, 2005).

TABLE 4
Anthocyanins* of black currant seed press residues (PR) and the test bread of human intervention study II (HIS II).

[mg/100 g]	Del3glc	Del3rut	Cy3glc	Cy3rut	Sum
PR used for HIS I	75.1	224	20.4	121	440
PR used for HIS II	33.5	81.9	9.7	48.3	173
Test bread for HIS II	0.42	1.03	0.24	2.03	3.72

Del3glc: Delphinidine-3-glucoside, Del3rut: Delphinidine-3-rutinoside, Cy3glc: Cyanidine-3-glucoside, Cy3rut: Cyanidine-3-rutinoside

* Analyses were conducted by the Anhalt University in the research group of Prof. Dr. I. Schellenberg.

The test bread used for HIS II contained 8% black currant PR. Thus, 13.9 $\text{mg}/100\text{ g}$ anthocyanins would be expected in the test bread. Instead, 3.72 $\text{mg}/100\text{ g}$ were measured, probably resulting from baking losses of up to 73% (TABLE 4). Studies show that concentrations of phenolic compounds in faecal water can be high (JENNER *et al.*, 2005). However, considering the relatively low intake and absorption rates of anthocyanins, absorption or recovery of anthocyanins in human samples were not tested in the both HIS (WU *et al.*, 2002).

The energy content of black currant PR was 20.7 MJ/100 g, which equates 4.14 MJ at a 20 g/d PR intervention. Thus, its energy contribution in the intervention studies is of minor importance.

Black currant PR for HIS II contained 25.7% oil which was much more than in the other tested PR, e.g. cranberry PR with 5.6% oil (MANUSCRIPT I - TABLE 1). The black currant PR contained 79% polyunsaturated fatty acids and an outstandingly high concentration of long-chain fatty acids (C15-C24) (MANUSCRIPT I - TABLE 2). The latter was characteristic for the entire PR tested. Stearidonic acid, effective in increasing tissue eicosapentaenoic acid concentration in human independently of $\Delta 6$ -desaturase, was from all tested PR only present in black currant PR (JAMES *et al.*, 2003). In addition, the co-occurrence of stearidonic acid and γ -linolenic acid present in black currant PR is very unique and only known for hemp seed oil, gooseberry seed oil and oil from the genus *Echium* (MATTHÄUS & BRÜHL, 2008).

The black currant PR had moderate amounts of vitamin C (41.7 mg/100 g PR for HIS II) (MANUSCRIPT I - FIGURE 2). Testing minerals showed that the iron concentration in black currant PR was high relative to other plants (MANUSCRIPT I). The amino acids were of medium quality (MANUSCRIPT I - TABLE 4). Resulting from the above described dominating substances in black currant PR, the tests in the both HIS focused on the antioxidant potential of the black currant PR, tocopherols and fibres biomarkers associated to those.

4.2 HUMAN INTERVENTION STUDIES – MAIN OUTCOMES

For consumption, at HIS I the black currant seed PR was applied in pure yoghurt (1.5% FDM). By the daily intervention of 10 g PR/d following 20 g PR/d no dose effects could be verified in the biomarkers measured, besides in the genotoxicity and cytotoxicity of faecal water, which was significantly higher in the 20 g PR/d dosage (TABLES A-2 to 5).

HIS II was conducted to include tests considering the PR when processed into a food. Therefore, the black currant seed PR was baked into whole grain bread. The bread was tested in 36 female subjects and compared to a control bread and baseline data (MANUSCRIPTS II and III). Different biomarkers were conducted to evaluate the far-reaching spectrum of postulated ways of action. The main effects are condensed in the following and in FIGURE 6.

4.2.1 FEATURES OF OXIDATIVE STRESS

➤ CYTO- AND GENOTOXICITY OF FAECAL WATER

The intervention of PR in yoghurt as well as in bread caused a multitude of adverse effects. The genotoxicity of faecal water reflected in the comet assay as well as the cytotoxicity of

faecal water significantly increased after the 20 g/d PR intervention in both of the applied matrices compared to the respective baseline data (MANUSCRIPT II - TABLE 4; TABLE A-3). Additionally, there was an increase from the 10 g/d to the 20 g/d PR intervention of the genotoxicity of faecal water (TABLE 1). A possible reason for that increase can be the increased PUFA intake by the PR intervention that can promote lipid peroxidation (MANUSCRIPTS I and II) (JENKINSON *et al.*, 1999).

➤ *HYDROXYLATION PRODUCTS OF SALICYLIC ACID IN STOOL (ANTIOXIDANT CAPACITY OF STOOL)*

The antioxidant capacity of stool measured by the hydroxylation products of salicylic acid was significantly decreased by PR bread intervention (MANUSCRIPT II - TABLE 4). In HIS I the antioxidant capacity of stool was not measured. Due to correlation analyses, it is assumed that at PR bread consumption the increased iron intake resulting in increased iron concentrations in stool has caused the decreased antioxidant capacity of stool by radical formation (MANUSCRIPT II).

Hydroxylation products in stool were significantly increased in smokers compared to non-smokers ($P=0.011$). With respect to biomarkers of oxidative stress, this was the only parameter with significant differences between smokers and non-smokers (MANUSCRIPT II).

➤ *URINARY 8-OXO-2'-DEOXYGUANOSINE (8-OXODG)*

The urinary 8-oxodG which increases in parallel to oxidative DNA-damage tended to increase at PR bread intervention but remained unchanged when applied in yoghurt ($P<0.1$) (MANUSCRIPT II - TABLE 2; TABLE A-4). The PR consumed in HIS I contained more DNA-protecting anthocyanins than the PR used in HIS II. Reviewing studies showed that 8-oxodG can be decreased particularly when different antioxidant compounds were applied in combination (MØLLER & LOFT, 2006). Analyses of human samples in regard to polyphenols were not conducted but could be responsible for this circumstance. Correlation analyses in HIS II allow the assumption, that the increased 8-oxodG concentration was caused by the increased iron concentration in stool, which may support the formation of radicals, which are then subjected to the body (MANUSCRIPT II) (LUCESOLI *et al.*, 1999; KNÖBEL *et al.*, 2007).

➤ *F2-ISOPROSTANES AND PROSTAGLANDIN F_{2α} METABOLITES*

Prostanes are markers of lipid peroxidation. Their formation has different causes, 8-iso-prostaglandin F_{2α} (PGF_{2α}) is formed non-enzymatically by radicals and 15-keto-dihydro-PGF_{2α} is formed by enzymes and inflammatory response.

The both determined prostanes in urine were significantly increased at the 20 g/d PR intervention compared to the 10 g/d PR intervention (TABLE A-4). They remained unchanged at HIS II (MANUSCRIPT II - TABLE 5). Noticeably, when building subgroups, the urinary 8-iso-PGF_{2α} excretion significantly increased in the non-users of hormonal contraceptives at test bread intervention in HIS II, while users of hormonal contraceptives were not affected by intervention (MANUSCRIPT II - TABLE 5). Additionally, when consuming test bread, both of the determined prostanes were significantly lower in the users of hormonal contraceptives compared to the non-users.

The general increase of the measured urinary PGF_{2α} concentrations was likely caused by the increased PUFA intake by PR which leads to increased plasma PGF_{2α} concentrations (MANUSCRIPT II) (WEINBERG *et al.*, 2001). For the differences between users and non-users of hormonal contraceptives, the antioxidant properties of estrogens can account for. Estrogens act as antioxidants by the up-regulation of the production of antioxidant enzymes (VIÑA *et al.*, 2006). The consequences of that fact were shown in ovariectomised rats, where estrogen replacement therapy completely prevented the otherwise increased peroxide production (BORRÁS *et al.*, 2003).

➤ *IRON PARAMETERS*

In HIS II, iron concentration in stool significantly increased for test bread consumption compared to baseline, probably due to increased iron intake (MANUSCRIPT II - TABLE 3). In HIS I, only iron intake but not excretion was significantly increased with PR consumption (TABLE A-2). At the same time, the serum ferritin concentration significantly decreased for test bread consumption and also for PR in yoghurt, both compared to baseline (MANUSCRIPT II - TABLE 3; TABLE 1). This circumstance indicates the situation of an iron deficiency existing in the body. Such effect could be entailed by the consumption of phytic acid present in the black currant PR, because phytic acid is known for chelating iron (TABLE 3) (GRAF *et al.*, 1987; MINIHANE & RIMBACH, 2002).

➤ TEAC AND VITAMIN C IN SERUM AND URINE

No significant changes were verified for TEAC values and vitamin C concentrations between control and test bread consumption, neither in serum nor in urine (MANUSCRIPT III - TABLE 3). For HIS I TEAC assay and vitamin C analysis was only carried out in urine, where no dose effects and also no difference between baseline and 20 g PR/d intervention were verified (TABLE A-4). Comparing test bread consumption and baseline values, urinary TEAC significantly increased and vitamin C tended to increase ($P < 0.1$). These increases at PR bread intervention can originate from the increased supply of tocopherol that replaces the utilised serum tocopherol instead of being regenerated with vitamin C (NIKI *et al.*, 1995). Therefore, serum vitamin C remains unspent and is finally excreted in urine (MANUSCRIPT III) (NYYSSÖNEN *et al.*, 1997).

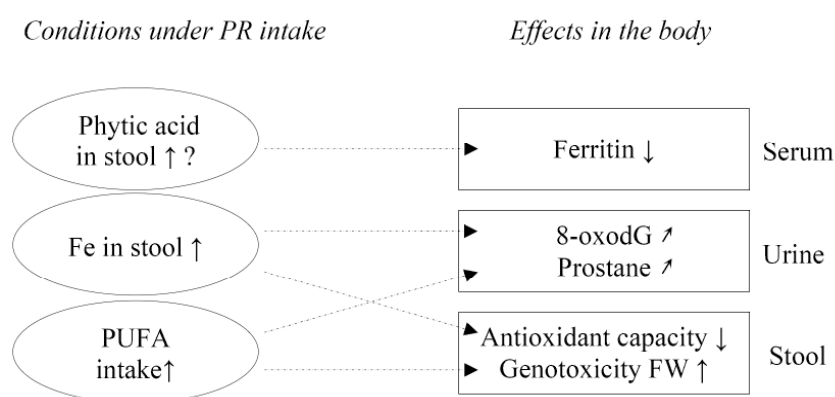


FIGURE 6 General changes and assumed effects in humans after consumption of black currant press residue baked into bread.

Effects were compared with control bread consumption (serum ferritin, urinary 8-oxodG, urinary prostanes, genotoxicity FW) or baseline data (antioxidant capacity in stool).

PR: Press residue

FW: Faecal water, 8-oxodG: 8-oxo-2'-deoxyguanosine

↑ Increase, ↓ Decrease, ↗ Increase in the subgroups smokers (8-oxodG) and non-users of hormonal contraceptive (prostate) ($P \leq 0.05$)

4.2.2 TOCOPHEROLS

Tocopherol was one of the most promising ingredients with health-promoting attributes present in the PR and was therefore considered in both HIS.

The intake of γ -tocopherol at PR consumption significantly increased in both HIS, α -tocopherol intake only significantly increased in HIS I (MANUSCRIPT III - TABLE 1;

TABLE A-2). In serum, particularly the serum γ -tocopherol concentration significantly increased when PR was consumed, but only in HIS II. In HIS I the serum tocopherol concentrations remained unchanged. To explain the effect of the increased serum γ -tocopherol concentration in HIS II, the tocopherol metabolism in the human body needs to be considered (FIGURE 7).

The usually consumed quantity of α - and γ -tocopherol is in about the same range but in favour of α -tocopherol though the relation depends on the diet (*e.g.* $\alpha:\gamma = 2:1$ in HIS II, control bread period). In blood instead, the ratio of $\alpha:\gamma$ -tocopherol concentration is about 20:1.

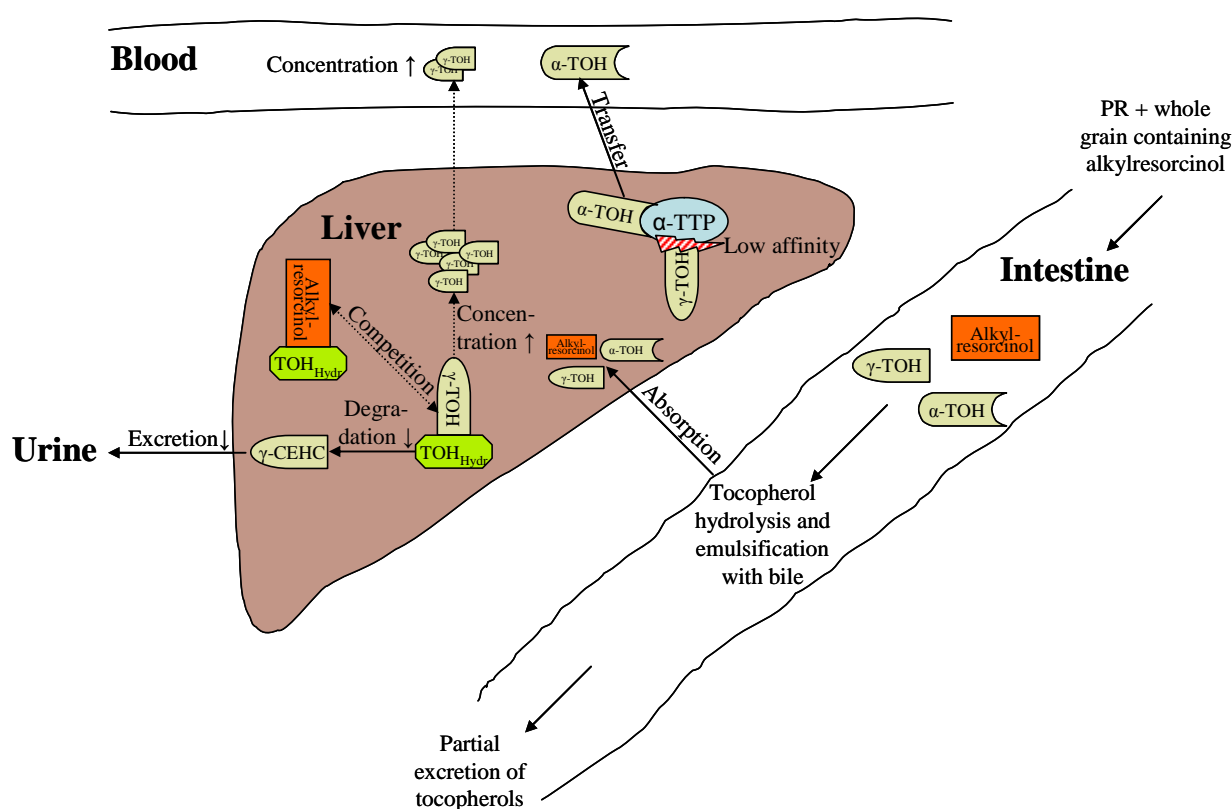


FIGURE 7 Interruption of γ -tocopherol metabolism by alkylresorcinol in the human body (according to SONNTAG & PARKER, 2002; KAEMPF-ROTZOLL *et al.*, 2003; ROSS *et al.*, 2004a).

PR: black currant seed press residue (tocopherol-rich); α -TOH: α -tocopherol; γ -TOH: γ -tocopherol; α -TTP: α -tocopherol-transfer-protein; CEHC: carboxyethyl-hydroxychromanol; TOH_{Hydr} : Tocopherol- ω -hydroxylase (cytochrome P450); Alkylresorcinol is a phenol found in wheat and rye husks.

After tocopherol intake the pancreatic enzymes hydrolyse the esterified tocopherol molecules and bile salts promote the emulsification into mixed micelles (SCHNEIDER, 2005). All the tocopherol isomers are equally incorporated into chylomicrons at the enterocyte. If not

transported to the liver directly, the chylomicrons reach peripheral tissues like adipose tissue or muscles (EGGERMONT, 2006). There, triacylglycerols are partially hydrolysed by lipolysis and tocopherol- and cholesterol-rich chylomicron remnants remain which are finally absorbed into the liver. At this point the α -tocopherol-transfer-protein (TTP) supports the transfer of α -tocopherol into the blood. γ -Tocopherol has a low affinity to α -TTP and therefore mainly remains in the liver (KAEMPF-ROTZOLL *et al.*, 2003). Only small quantities can be recovered in plasma. Its general metabolic pathway is to be degraded to γ -CEHC, which is hydrophilic and can be excreted *via* urine. The degradation is carried out by the enzyme cytochrome P450. This enzyme also degrades the phenolic, amphiphilic substance alkylresorcinol found *e.g.* in breads containing whole grain wheat or rye (ROSS *et al.*, 2003; ROSS *et al.*, 2004b). Consequently, competition for the degradation enzyme exists leading to enrichment of γ -tocopherol in liver and as a consequence in blood also.

The effect could not be shown with the consumption of control bread, though consumption of that bread also forced a significantly higher γ -tocopherol intake than at baseline (MANUSCRIPT III - TABLE 1). In this matter it can be assumed, that ingested γ -tocopherol amounts were too low to show an impact. Substances like sesamine, which works similarly, but more effective than alkylresorcinol, also need increased γ -tocopherol intake to show significant effects (FRANK *et al.*, 2005). Likewise, no effect was measured regarding the serum γ -tocopherol concentration in HIS I for 10 and 20 g/d intervention (TABLE A-5). Consequently, a special compound only found in the bread together with increased γ -tocopherol intake must cause the increase in serum γ -tocopherol concentration which would explain the unchanged serum γ -tocopherol concentrations in HIS I.

In a comparable study to HIS II involving 40 female students, an intervention with moderate α -tocopherol intakes together with γ -tocopherol intakes of different concentrations (additionally to a normal diet 9.5 mg/d and 24.1 mg/d, respectively) increased the serum γ -tocopherol concentration significantly, but lower doses of 5.4 mg γ -tocopherol/d failed to increase (LEMCKE-NOROJÄRVI *et al.*, 2001). At the present PR bread study instead, the comparatively low γ -tocopherol intervention dose of 2.0 mg/d was highly effective and forced an increased serum γ -tocopherol concentration.

However, by means of correlation analyses no major effects could be verified owing to the changes in serum tocopherol concentration at test bread consumption (MANUSCRIPT II). Nevertheless, the increased serum γ -tocopherol concentration could be beneficial in general, *e.g.* reduction of DNA damage or lipid peroxidation, but these benefits could be overlapped by negatively acting components of the PR (WINKLHOFFER-ROOB *et al.*, 2003).

The α -tocopherol intake in HIS I increased with 20 g/d PR intake, but it was not when applied in bread (TABLE A-2). The latter must be due to the fact that α -tocopherol-rich dishes consumed during the normal diet were substituted by the study bread (MANUSCRIPT III). Despite the increased α -tocopherol intake in HIS I, serum α -tocopherol concentration did not increase (TABLE A-5). Again, the tocopherol metabolism might explain: Unlike other lipophilic vitamins, tocopherols are not accumulated to a toxic level in the body but are metabolised (MUSTACICH *et al.*, 2006). Studies indicate that after a supplementation of 50-150 mg/d there is an upper threshold of α -tocopherol concentration in serum of 7-9 mmol/g total lipids (SCHULTZ *et al.*, 1995). Exceeding this level leads to the formation and excretion of α -CEHC. However, volunteers of the present study did not undergo such high tocopherol intakes (MANUSCRIPT III - TABLE 1; TABLE A-2). Another remarkable effect of excessive tocopherol administration is that serum tocopherol concentration is not distinctly altered, but tocopherol circulating in the body is replaced by newly ingested tocopherol (KAYDEN & TRABER, 1993).

Between smokers and non-smokers, there were no differences in serum tocopherol concentrations. Interestingly, for PR bread consumption non-smokers excreted significantly less tocopherols than smokers did. It indicates that smokers had either a reduced absorption or an increased bile excretion of the tocopherols, or non-smokers had a more efficient absorption of tocopherols followed by increased metabolism to CEHC (MANUSCRIPT III).

4.2.3 FIBRE

Fibre intake is associated with lowered serum cholesterol concentrations (ANDERSON *et al.*, 1984; SHEN *et al.*, 1998; CHAU *et al.*, 2004). Studies suggest that the binding abilities of certain fibres to faecal bile acids or cholesterol account for it (EBIHARA & SCHNEEMAN, 1989; BOREL *et al.*, 1990; CHAPLIN, 2003). This instance results in an interruption of the enterohepatic circulation affecting the cholesterol metabolism. Therefore, also serum lipid parameters and neutral sterols in faeces were analysed in the human intervention studies.

PR consumption in both studies was accompanied by significantly increased fibre intakes but had no influences on serum cholesterol or the HDL/LDL ratio (MANUSCRIPT III; TABLES A-2, A-5 and A-6). A study using fibre in rats also showed that wheat bran and germ has little impact on the intestinal lipid absorption (BOREL *et al.*, 1990). These fibres bind between 7-15% lipids and cholesterol but are without impact on serum lipids and cholesterol.

However, potential effects are always linked to the type of fibre used (DONGOWSKI *et al.*, 2002).

Faecal short-chain fatty acids (SCFA) and pH-value are biomarkers of the metabolic activity of the faecal microbiota (CUMMINGS & MACFARLANE, 2002). Besides significantly increased *i*-valerate excretion with control and test bread, no significant changes in the concentration of total SCFA were verified (TABLE A-7). At the HIS I, with consuming the same amount (20 g/d) black currant PR in 125 g yoghurt/d instead of in bread, acetate, *n*-butyrate and total SCFA were significantly increased compared to baseline with the pure yoghurt only (TABLE A-3). Thus, fermentation or specific bacteria colonisation took only place in PR associated to yoghurt, not to bread.

The neutral sterols in stool are metabolites of the faecal cholesterol and are associated to fibre intake (REDDY *et al.*, 1989). Depending on the type of fibre, neutral sterol excretion increases (oat) or decreases (wheat bran, cellulose). There were no differences between 10 g and 20 g PR intervention in faecal neutral sterol excretion, but both doses significantly decreased the excretion when compared to baseline (TABLE A-3). When applied in bread, a highly significant decrease of faecal neutral sterol excretion was noticed only due to the bread consumption but not to the PR additionally (TABLE A-7).

4.3 DISCUSSION OF METHODS

To extensively evaluate the PR of berry seeds, a wide spectrum of analyses was conducted (MANUSCRIPT I; TABLES 2 to 4). Additionally, their physiological effect at consumption was evaluated using two human intervention studies, again employing a multitude of analyses by means of the matrices stool, urine and blood (MANUSCRIPTS II and III; TABLES A-2 to A-7). The large-scale testing covered effects on the antioxidant capacity of serum, stool and urine (TEAC, DHBA formation), cytotoxicity and genotoxicity of faecal water (comet assay), lipid peroxidation in the body (8-iso-PGF_{2α} and 15-keto-dihydro-PGF_{2α} in urine), DNA damage of the whole body (8-oxodG in urine), micronutrients in serum, stool and urine (vitamin C, tocopherols), faecal fibre concentration, serum iron parameters and further minor components. Generally, all methods contemplated solely can suggest different outcomes compared to when looking at the results as a whole. For example, increased tocopherol concentrations in serum and faeces would suggest increased antioxidant defence leading to less oxidative damage. Instead, 8-oxodG, genotoxicity and cytotoxicity of faecal water and DHBAs implicate that oxidative damage was increased (MANUSCRIPT II). Overall, the faecal matrix is very complex

and effects can result as a combined effect of different substances. Consequently, effects cannot always be attributed to one single substance present in the gastrointestinal tract. Furthermore, methods not measuring substances present in a matrix directly but by indirect effects may skew the final outcome (*e.g.* DHBA formation, comet assay). Hence, it cannot always be assured that reactions claimed were caused by the designated substances, or if reactions underlie external interferences, *e.g.* oxygen in the system, or finally, if the *in vitro* model is relevant for physiological conditions at all. After all, the wide spectrum of analyses in the human studies allowed a general evaluation of the physiological effects after black currant PR consumption in regard to health-promoting abilities.

The measured effects after consumption of black currant PR cannot simply be generalised for the PR of other berry seed species, because the PR of different species were not similar in their composition (MANUSCRIPT I).

The both studies and hence results have some general limitations. The participants involved were exclusively women and were all narrow aged. The studies comprised no cross-over design, all participants consumed control bread first and subsequently the test bread. Though an identical standardised diet was administered for sample collection at each period, the participants could not warrant for identical food intake between periods. The explanation for the increased serum γ -tocopherol concentration, one of the main outcomes of the HIS II, can be substantiated if the concentrations of the γ -tocopherol metabolite γ -CEHC are measured in urine and serum.

4.4 CONCLUSIONS

The PR by itself bears promising, health-promoting ingredients, like the tocopherols or the fibre. Nevertheless, some of those ingredients can have changed unfavourably by the seed processing to the PR, for example, lipid peroxidation can proceed due to the heat development during the press procedure.

Generally, testing complex food matrices makes it difficult to obtain clear and distinct outcomes compared with single intervention substances. It was found out that the matrix, which PR was applied with, influenced the effects of intervention.

Black currant PR consumption shows adverse effects with advantages, *e.g.* increased serum γ -tocopherol concentration, but also disadvantages, *e.g.* decreased antioxidant capacity of stool and increased genotoxicity and cytotoxicity of faecal water. Finally, the disadvantages dominate over the advantages. Therefore, the application of black currant PR in terms of a

functional food is not to be recommended. For PR from other berry species neither general nor evident conclusions can be drawn regarding their physiological effects after consumption. Though the species are closely related, their ingredients are too different from each other.

Besides the main feature to evaluate the PR as potential functional food ingredient, effects found in the human studies suggest that an alternative strategy to improve the γ -tocopherol concentration in serum was to enrich whole grain bread with γ -tocopherol. These considerations base on the fact that at the combination of increased γ -tocopherol intake in parallel with whole grain intake the serum γ -tocopherol concentration increased. Even more, the option wins importance because at vitamin E supplementation mainly α -tocopherol is utilised instead of making effort of the potential of other tocopherol isomers. Yet worse, large doses of α -tocopherol reduce the serum concentration of γ -tocopherol and therewith the potential of the many benefits of γ -tocopherol.

The conducted work supports the claim of physiologists that nutrients and substances aimed to be nutrients must not be analysed exclusively isolated for their final evaluation. In fact, nutrients must be understood in the context of a balanced diet.

SUMMARY
(ENGLISH AND GERMAN)

5 SUMMARY

BACKGROUND Black currant berries are utilised in many food products. Mostly, the seeds remain as waste product, though it can be assumed that the seeds contain appreciated substances similar to those found in the berry pulp. To exploit the full potential of the berry fruits, also berry seeds and their seed press residues should attract attention in regard to their health-promoting substances.

OBJECTIVE This work was considered to analyse the ingredients of press residues (PR) of berry seeds and to verify their potential to serve as a functional food. Thereby the focus was on black currant PR. The analyses also included PR of bilberry (*Vaccinium myrtillus* L.), cranberry (*Vaccinium oxycoccus* L.), rose hip (*Rosa canina* L.), strawberry (*Fragaria x ananassa* L.), elder (*Sambucus nigra* L.), and two batches of black currant (*Ribes nigrum* L.). Further, the physiological effects of black currant PR consumption were to be evaluated.

METHODS All PR were analysed for their content of oil, fatty acid pattern, concentrations of tocopherols, tocotrienols, vitamin C, total fibre, as well as regarding their antioxidant capacity and gallic acid equivalent. Furthermore, in black currant PR concentrations of the fibre fractions NDF and ADF, of inositol phosphates, anthocyanins, minerals, plant sterols, carotenoids, proteins and their quality, as well as dry matter, ashes and energy content were analysed. To test black currant PR with regard to substances that seemed to be promising considering their health-beneficial potential, two human intervention studies were carried out. As main ingredients of the PR, tocopherols and fibre were defined.

The both human intervention studies were conducted in the same design, each including three periods. All participants started with a five-day baseline period without intervention for baseline-data acquisition. This was followed by two four-week periods. The human intervention study I was conducted with 18 female participants to test dose effects. The intervention dose was 10 g PR/d followed by 20 g PR/d, both doses in yoghurt. To test the PR in a processed food, the human intervention study II was conducted including 36 female participants. PR was incorporated into bread because this food provided a neutral and appropriate matrix for a convenient PR intake. In accordance with the results of the study I, in the study II the dose of 20 g PR/d was chosen which resulted in a bread intake of 250 g/d for

four weeks. The test bread was compared to a control bread without PR. Stool, urine and blood samples were collected during a five-day standardised diet with equal conditions between each period. Following parameters were determined: Tocopherol of serum and stool, urinary 8-oxo-2'-deoxyguanosine, antioxidant capacity of stool, cyto- and genotoxicity of faecal water, F₂-isoprostanes and prostaglandin F_{2α} metabolites, vitamin C and antioxidant capacity in serum and urine, fibre, neutral sterols and short-chain fatty acids in stool.

RESULTS

The investigation of berry seed PR revealed that tocopherols and fibre were the quantitatively most important substances of this test material (e.g. black currant 17.5 ± 1.1 mg tocopherol/100 g PR). However, there were significant differences between batches and species of the PR. All PR had a high PUFA content (>65%) and a favourable *n*-6/*n*-3 fatty acid ratio (<3.6:1).

No general differences could be verified between the both applied doses of black currant PR except increased fibre and tocopherol intakes and increased genotoxicity and cytotoxicity of faecal water for the 20 g/d dose compared to the 10 g/d dose ($P < 0.05$). From baseline to 10 g intervention the increase of genotoxicity of faecal water and the decrease of neutral sterol excretion were also significant ($P < 0.05$).

In the study II, after PR bread consumption the total tocopherol concentration increased in serum vs. control bread and in stool vs. baseline ($P < 0.05$). The combined consumption of whole grain bread and PR led to increased serum β - and γ -tocopherol concentrations ($P < 0.001$). However, neither bread alone nor PR in yoghurt had such implications. This effect can be due to alkylresorcinol found in wheat and rye of the whole grain bread. It competes together with γ -tocopherol for their degradation enzyme leading to reduced γ -tocopherol metabolism resulting in γ -tocopherol accumulation. Further, compared to control bread, after the consumption of PR bread the faecal iron concentration increased, at the same time the serum ferritin concentration decreased ($P < 0.05$). Through PR bread the cyto- and genotoxicity of faecal water increased with the parallel reduction of the antioxidant capacity in stool ($P < 0.05$). The concentration of vitamin C and the antioxidant capacity in urine increased after PR bread intervention ($P < 0.05$); the urinary 8-oxo-2'-deoxyguanosine concentration tended to increase ($P < 0.1$). The faecal fibre concentration significantly increased after PR bread intervention ($P < 0.05$). Independently of the intervention, the daily excretion of fibre was closely correlated to the daily excretion of each tocopherol isomer in stool ($P < 0.001$).

CONCLUSION Consumption of black currant PR-enriched bread for four weeks had adverse effects on the antioxidant status of the participants, whilst serum and stool total tocopherol concentrations were increased. The tocopherol in serum and stool had no effect on the measured biomarkers associated with oxidative stress. Due to the adverse effects of black currant PR, its application in terms of a functional food cannot be recommended.

ZUSAMMENFASSUNG

HINTERGRUND Schwarze Johannisbeeren finden in vielen Lebensmitteln Verwendung. Dabei verbleiben die Samen zumeist als Abfallprodukt, obwohl in den Samen wertvolle Inhaltsstoffe ähnlich denen des Fruchtfleisches zu vermuten sind. Um das gesamte nutritive Potential der Beerenfrüchte auszuschöpfen, sollten auch die Samen und daraus gewonnene Pressrückstände auf gesundheitsfördernde Substanzen untersucht werden.

ZIELSETZUNG Die Arbeit wurde mit dem Ziel durchgeführt, die Inhaltsstoffe der Pressrückstände (PR) von Beerensamen zu analysieren und deren Potential für funktionelle Lebensmittel zu prüfen. Der Fokus lag dabei auf dem PR der Schwarzen Johannisbeere. In die Untersuchungen einbezogen waren PR aus Heidelbeere (*Vaccinium myrtillus* L.), Preiselbeere (*Vaccinium oxycoccus* L.), Hagebutte (*Rosa canina* L.), Erdbeere (*Fragaria x ananassa* L.), Holunder (*Sambucus nigra* L.) sowie zwei verschiedene Chargen der Schwarzen Johannisbeere (*Ribes nigrum* L.). Weiterhin sollten die physiologischen Effekte des Verzehr von PR der Schwarzen Johannisbeere beurteilt werden.

METHODEN Alle PR wurden auf ihren Gehalt an Öl, ihre Fettsäurenverteilung, ihre Konzentrationen an Tocopherolen, Tocotrienolen, Vitamin C und Gesamtballaststoffen sowie hinsichtlich antioxidativer Kapazität und Gallussäureäquivalent untersucht. Im PR der Schwarzen Johannisbeere wurden zusätzlich die Konzentrationen der Faserfraktionen NDF und ADF, der Inositolphosphate, Anthocyane, Mineralstoffe, Phytosterole, Carotinoide, des Eiweißes einschließlich Eiweißqualität, sowie die Trockenmasse, der Asche- und Energiegehalt bestimmt.

Die Prüfung des PR der Schwarzen Johannisbeere im Hinblick auf Inhaltstoffe, die bezüglich des gesundheitsfördernden Potentials am aussichtsreichsten erschienen, erfolgte mittels zweier Humaninterventionsstudien. Als wertgebende Substanzen des PR wurden Tocopherole und Ballaststoffe definiert.

Die beiden Humaninterventionsstudien bestehend aus je drei Perioden waren im gleichen Design angelegt. Jede Probandin startete mit einer fünftägigen Baseline-Periode zur Erfassung der Basisdaten. Es folgten zwei vierwöchige Interventionsperioden. Die Humaninterventionsstudie I wurde mit 18 Probandinnen zur Untersuchung von Dosiseffekten vorgenommen. Die Intervention betrug 10 g PR/d, gefolgt von 20 g PR/d, jeweils in Joghurt.

Um den PR in einem verarbeiteten Lebensmittel zu untersuchen, wurde eine Humaninterventionsstudie II mit 36 Probandinnen durchgeführt. Der PR wurde in Brot eingearbeitet, da dieses Lebensmittel eine geeignete, neutrale Matrix für den PR-Verzehr bietet. Entsprechend den Ergebnissen der Studie I wurde für die Studie II eine Dosis von 20 g PR/d gewählt, was mit einer täglichen Brotaufnahme von 250 g über vier Wochen einherging. Das Testbrot wurde mit einem Kontrollbrot ohne PR verglichen. Stuhl-, Urin- und Blutproben wurden unter festgelegten Bedingungen an den letzten fünf Tagen jeder Periode bei standardisierter Kost gewonnen. Folgende Parameter wurden bestimmt: Tocopherol im Serum und Stuhl, 8-Oxo-2'-deoxyguanosin im Urin, antioxidative Kapazität des Stuhls, Cyto- und Genotoxizität des Fäzeswassers, F₂-Isoprostane und Prostaglandin-F_{2α}-Metabolite, Vitamin C und antioxidative Kapazität im Serum und Urin, Ballaststoffe, neutrale Sterole und kurzkettige Fettsäuren im Stuhl.

ERGEBNISSE Die Untersuchungen der Beerensamen PR zeigten, dass Tocopherole und Ballaststoffe die quantitativ bedeutendsten Inhaltsstoffe der Testmaterialien darstellten (z. B. Schwarze Johannisbeere $17,5 \pm 1,1$ mg Tocopherol/100 g PR). Allerdings gab es deutliche Unterschiede zwischen den Chargen und Spezies der PR. Alle PR wiesen einen hohen PUFA-Gehalt (> 65%) sowie ein physiologisch günstiges Verhältnis von *n*-6/*n*-3-Fettsäuren (< 3,6 : 1) auf.

Zwischen den beiden verabreichten Dosen des PR der Schwarzen Johannisbeere konnten keine generellen Unterschiede festgestellt werden, ausgenommen die erhöhte Ballaststoff- und Tocopherolaufnahme und die erhöhte Geno- und Cytotoxizität des Fäzeswassers bei der 20 g PR/d-Dosis verglichen mit der 10 g PR/d-Dosis ($P < 0,05$). Von Baseline-Periode zu 10 g-Intervention waren auch der Anstieg der Genotoxizität des Fäzeswassers sowie der Abfall der Ausscheidung der neutralen Sterole signifikant ($P < 0,05$).

In der Studie II erhöhte sich durch den PR-Brot-Verzehr die Konzentration des Gesamttocopherols im Serum verglichen mit dem Kontrollbrot bzw. im Stuhl verglichen mit der Baseline-Periode ($P < 0,05$). Der parallele Verzehr von Vollkornbrot zusammen mit PR führte im Serum zu erhöhten β - und γ -Tocopherol-Konzentrationen ($P < 0,001$). Hingegen konnten weder Brot allein noch der PR in Joghurt eine solche Wirkung hervorrufen. Dieser Effekt könnte durch Alkyresorcinol des Weizens und Roggens im Vollkornbrot bedingt sein. Zusammen mit γ -Tocopherol konkurriert es um ein Enzym, welches das γ -Tocopherol metabolisiert. Dadurch wird γ -Tocopherol vermindert abgebaut und es erfolgt eine γ -Tocopherol-Akkumulation. Des Weiteren führte, jeweils verglichen mit dem Kontrollbrot,

der PR-Brot-Verzehr zur erhöhten Eisenausscheidung im Stuhl, gleichzeitig verringerte sich die Serum-Ferritin-Konzentration ($P < 0,05$). Die Cyto- und Genotoxizität des Fäzeswassers erhöhte sich durch PR-Brot, während sich die antioxidative Kapazität im Stuhl reduzierte ($P < 0,05$). Die Ausscheidung an Vitamin C sowie die antioxidative Kapazität im Urin stiegen nach PR-Brot-Verzehr an ($P < 0,05$); für den Anstieg der Ausscheidung an 8-Oxo-2'-deoxyguanosin im Urin bestand ein Trend ($P < 0,1$). Die Konzentration an Gesamtballaststoffen im Stuhl erhöhte sich durch die PR-Brot-Intervention signifikant ($P < 0,05$). Unabhängig von der Intervention korrelierte die jeweilige tägliche Ausscheidung an Gesamtballaststoffen mit den einzelnen Tocopherolisomeren im Stuhl ($P < 0,001$).

SCHLUSSFOLGERUNG Der vierwöchige Verzehr von Brot, angereichert mit PR der Schwarzen Johannisbeere, wirkt sich nachteilig auf den antioxidativen Status der Probanden aus, während die Gesamttocopherolkonzentrationen im Serum und Stuhl erhöht sind. Die Tocopherolkonzentrationen im Serum und Stuhl hatten keinen Einfluss auf die untersuchten Biomarker des oxidativen Stresses. Aufgrund nachteiliger Effekte des PR der Schwarzen Johannisbeere kann dessen Anwendung in einem funktionellen Lebensmittel nicht empfohlen werden.

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6 REFERENCES

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APPENDIX

TABLE A-1

Foods provided during a 5-day standardised diet (intervention study II, test bread period)

Day 1	Day 2	Day 3	Day 4	Day 5
Test bread (250 g)	Test bread (250 g)	Test bread (250 g)	Test bread (250 g)	Test bread (250 g)
Butter (20 g)	Butter (20 g)	Butter (20 g)	Butter (20 g)	Butter (20 g)
Edam cheese 45% FDM (40 g)	Edam cheese 45% FDM (40 g)	Edam cheese 45% FDM (40 g)	Camembert 60% FDM (40 g)	Edam cheese 45% FDM (40 g)
Cream cheese with herbs (16 g)	Cream cheese (16 g)	Cream cheese (16 g)	Cream cheese with herbs (16 g)	Camembert 60% FDM (40 g)
-	-	-	-	Curd cheese with herbs (62.5 g)
Boiled ham (25 g)	Ring bologna from poultry (25 g)	Cervelat sausage (25 g)	Boiled ham (15 g)	Ring bologna from poultry (15 g)
-	-	-	Cervelat sausage (12 g)	Cervelat sausage (12 g)
Jam wild berry (25 g)	Jam cherry (25 g)	Jam apricot (25 g)	Chocolate spread (20 g)	Jam strawberry (25 g)
-	-	-	-	Honey (20 g)
Cornflakes (30 g)	-	Yoghurt 3.5% (150 g)	-	Yoghurt 3.5% (150 g)
Pear (150 g)	Pear (150 g)	Apple (150 g)	Apple (150 g)	Pear (150 g)
-	-	Banana (200 g)	Banana (200 g)	Kiwi (60 g)
-	Apple (150 g)	Kiwi (60 g)	-	Orange (120 g)
Tomato (120 g)	Carrots (100 g)	Carrots (100 g)	Cucumber (150 g)	Tomato (120 g)
Cucumber (150 g)	Paprika (100 g)	Tomato (120 g)	Red pepper (100 g)	Cucumber (150 g)
Chocolate bar with milk cream (25 g)	Chocolate bar with milk cream (25 g)	Sandwich biscuit (37,5 g)	Fruit gums (35 g)	Hazelnut waffle (25 g)
Sugar (10 g)	Sugar (10 g)	Sugar (10 g)	Sugar (10 g)	Sugar (10 g)
Chicken filet & noodles (460 g)	Fish pan and potatoes (550 g)	Spaghetti Bolognese (550 g)	Curd cheese fritters, served sweet (430 g)	Vegetable stew (600 g)
Milk 3.5% (200 ml)	Milk 3.5% (200 ml)	Milk 3.5% (200 ml)	Milk 3.5% (200 ml)	Milk 3.5% (200 ml)
Apple juice (200 ml)	Orange juice (200 ml)	Apple juice (200 ml)	Apple juice (200 ml)	Orange juice (200 ml)
Further beverages: black tea, peppermint tea, fruit tea (as 2 tea bags/day) and mineral water (<i>ad lib.</i>)				

Food provided was based on an energy intake of 10 MJ per day.

TABLE A-2

Food intake¹ and parameters of the faecal matrix during a 5-day standardised diet with 3-day stool collection following consumption of black currant seed press residue (PR) in yoghurt (means \pm SD; $n = 18$).

Parameters	Unit	Baseline	10 g PR/d	20 g PR/d
<i>Intake</i>				
Energy ¹	[MJ/d]	7.47 \pm 0.92	7.51 \pm 0.91	7.13 \pm 1.38
Fat ¹	[g/d]	68.8 \pm 11.9	67.9 \pm 10.2	64.5 \pm 13.2
Protein ¹	[g/d]	69.0 \pm 8.7	68.2 \pm 8.0	64.1 \pm 12.0
Carbohydrate ¹	[g/d]	217 \pm 22	221 \pm 27	211 \pm 44
Fibre ²	[g/d]	27.7 \pm 3.9 ^c	32.4 \pm 5.1 ^b	35.4 \pm 6.8 ^a
Vitamin C ¹	[mg/d]	139 \pm 27 ^a	134 \pm 32 ^a	118 \pm 35 ^b
Iron ¹	[mg/d]	9.2 \pm 1.1 ^c	11.2 \pm 1.2 ^b	12.5 \pm 1.9 ^a
Tocopherol α	[μ mol/d]	11.5 \pm 1.3 ^c	12.7 \pm 1.8 ^b	14.6 \pm 2.3 ^a
β		1.42 \pm 0.16	1.46 \pm 0.29	1.42 \pm 0.39
γ		3.50 \pm 0.34 ^c	5.37 \pm 0.61 ^b	7.01 \pm 0.75 ^a
total		16.4 \pm 1.7 ^c	19.5 \pm 2.4 ^b	23.0 \pm 3.3 ^a
<i>Excretion</i>				
Fibre	[g/100 g]	8.31 \pm 1.76	8.43 \pm 2.36	8.57 \pm 2.84
Iron	[mg/d]	10.2 \pm 10.1	11.6 \pm 11.3	10.6 \pm 8.2
Tocopherol α	[μ mol/d]	12.6 \pm 5.4	12.5 \pm 5.6	13.8 \pm 7.3
β		3.01 \pm 1.35	2.88 \pm 1.93	2.34 \pm 1.19
γ		2.19 \pm 1.30 ^b	3.75 \pm 2.74 ^a	3.93 \pm 2.05 ^a
total		17.8 \pm 7.6	19.1 \pm 9.9	20.1 \pm 10.4
Stool mass	[g/d]	110 \pm 41	116 \pm 48	116 \pm 36
Dry matter stool	[%]	25.9 \pm 5.1	25.8 \pm 5.2	24.7 \pm 5.3
pH-value stool		6.71 \pm 0.42 ^a	6.63 \pm 0.37 ^{a,b}	6.43 \pm 0.55 ^b

^{a,b,c} Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

Statistical evaluation was carried out using analysis of variance for repeated measures (SPSS 14.0 software package, SPSS Inc., Chicago, IL, USA).

¹ Calculated using Prodi® 5.4 software.

² Fibre concentrations of foods were analysed at institute's laboratory.

TABLE A-3

Concentration of the faecal short-chain fatty acids, neutral sterols and cyto- and genotoxicity of faecal water (comet assay, given in fluorescence tail intensity) during a 5-day standardised diet with 3-day stool collection following consumption of black currant seed press residue (PR) in yoghurt (means \pm SD; $n = 18$).

Parameters	Baseline	10 g PR/d	20 g PR/d
[$\mu\text{mol/g}$]			
Total short-chain fatty acids	74.9 \pm 23.5 ^b	89.4 \pm 29.4 ^{a,b}	95.1 \pm 28.8 ^a
Acetate	43.5 \pm 13.9 ^b	53.4 \pm 19.9 ^a	56.1 \pm 16.8 ^a
Propionate	14.0 \pm 5.4	16.7 \pm 5.4	16.4 \pm 5.3
<i>i</i> -Butyrate	1.70 \pm 0.66	1.81 \pm 0.82	1.61 \pm 0.53
<i>n</i> -Butyrate	10.6 \pm 5.1 ^b	12.4 \pm 5.3 ^{a,b}	16.1 \pm 8.7 ^a
<i>i</i> -Valerate	2.39 \pm 1.04	2.50 \pm 1.15	2.11 \pm 0.78
<i>n</i> -Valerate	1.99 \pm 0.73	2.04 \pm 0.58	2.11 \pm 0.59
<i>n</i> -Capronate	0.68 \pm 0.77	0.56 \pm 0.69	0.63 \pm 0.73
[mg/g fresh matter]			
Total neutral sterols	7.39 \pm 5.04 ^a	6.00 \pm 4.42 ^b	5.16 \pm 3.22 ^b
Coprostanol	3.81 \pm 3.71	3.23 \pm 2.68	2.69 \pm 2.06
Cholesterol [†]	2.77 \pm 5.14	2.20 \pm 3.88	1.95 \pm 2.99
Coprostanon	0.378 \pm 0.555	0.255 \pm 0.284	0.277 \pm 0.290
Cholestanol [†]	0.424 \pm 0.968	0.319 \pm 0.712	0.241 \pm 0.337
Cholestanon [†]	0.006 \pm 0.014	0.004 \pm 0.009	0.004 \pm 0.006
[%]			
Cell viability	97.7 \pm 2.1 ^a	97.4 \pm 3.5 ^a	95.1 \pm 4.6 ^b
Comet assay	12.7 \pm 5.7 ^c	15.9 \pm 8.8 ^b	22.5 \pm 14.9 ^a

^{a,b,c} Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

Statistical evaluation was carried out using analysis of variance for repeated measures (SPSS 14.0 software package, SPSS Inc., Chicago, IL, USA).

[†] Values were not distributed normally; significance was calculated by means of the Wilcoxon test.

TABLE A-4

Concentrations of urinary trolox equivalent antioxidant capacity (TEAC), vitamin C, 8-oxo-2'-deoxyguanosine (8-oxodG), 8-iso-Prostaglandin F_{2α} and 15-keto-dihydro-Prostaglandin F_{2α} during a 5-day standardised diet with 3-day urine collection following consumption of black currant seed press residue (PR) in yoghurt (means ± SD; *n* = 18).

Parameters	Unit	Baseline	10 g PR/d	20 g PR/d
TEAC urine	[mmol/l]	4.73 ± 3.21	4.35 ± 2.58	4.90 ± 2.57
Vitamin C urine	[μmol/d]	350 ± 239	317 ± 223	307 ± 219
8-oxodG	[nmol/kg/24 h]	1.63 ± 2.50	1.35 ± 2.11	1.37 ± 1.65
8-iso-Prostaglandin F _{2α}	[nmol/24 h]	n.a.	6.68 ± 3.50 ^b	9.96 ± 5.17 ^a
15-keto-dihydro-Prostaglandin F _{2α}	[nmol/24 h]	n.a.	1.81 ± 0.92 ^b	2.47 ± 1.45 ^a

^{a,b} Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

Statistical evaluation was carried out using analysis of variance for repeated measures (SPSS 14.0 software package, SPSS Inc., Chicago, IL, USA).

n.a.: not analysed

TABLE A-5

Concentration of lipids, tocopherols and iron parameters in serum during a 5-day standardised diet following consumption of black currant seed press residue (PR) in yoghurt (means \pm SD; $n = 18$).

Parameters		Unit	Baseline	10 g PR/d	20 g PR/d
Total cholesterol		[mmol/l]	4.63 \pm 0.89	4.59 \pm 1.00	4.51 \pm 0.95
HDL cholesterol		[mmol/l]	1.68 \pm 0.49 ^a	1.62 \pm 0.45 ^a	1.53 \pm 0.41 ^b
LDL cholesterol		[mmol/l]	3.12 \pm 0.96 ^a	2.95 \pm 1.09 ^a	2.73 \pm 0.98 ^b
LDL/HDL cholesterol			2.02 \pm 0.97	2.01 \pm 1.17	1.93 \pm 0.99
Triacylglycerol		[mmol/l]	1.01 \pm 0.49	1.00 \pm 0.55	1.13 \pm 0.58
Tocopherol	α	[μ mol/l]	30.3 \pm 6.2	29.3 \pm 6.1	29.7 \pm 7.6
	β		0.53 \pm 0.10	0.49 \pm 0.13	0.51 \pm 0.13
	γ		1.22 \pm 0.37	1.27 \pm 0.34	1.34 \pm 0.42
	total		32.1 \pm 6.6	31.1 \pm 6.4	31.5 \pm 8.0
Tocopherol (lipid-adjusted)	α	[μ mol/mmol serum lipids]	5.45 \pm 0.85	5.34 \pm 0.90	5.26 \pm 0.83
	β		0.095 \pm 0.018 ^a	0.091 \pm 0.028 ^{a,b}	0.090 \pm 0.018 ^b
	γ		0.22 \pm 0.05	0.24 \pm 0.08	0.24 \pm 0.07
	total		5.76 \pm 0.90	5.67 \pm 0.98	5.59 \pm 0.88
Iron in serum		[mg/l]	19.3 \pm 12.5	17.5 \pm 8.9	14.5 \pm 6.3
Ferritin		[μ g/l]	45.8 \pm 33.2 ^a	38.4 \pm 25.2 ^b	36.8 \pm 26.2 ^b
Transferrin		[g/l]	3.17 \pm 0.50 ^b	3.31 \pm 0.50 ^a	3.45 \pm 0.62 ^a
Transferrin saturation		[%]	25.5 \pm 18.3	21.8 \pm 12.0	17.4 \pm 8.5

^{a,b} Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

Serum lipids were achieved using the Synchron[®] LX 20 (BECKMAN Coulter, Brea CA).

Statistical evaluation was carried out using analysis of variance for repeated measures (SPSS 14.0 software package, SPSS Inc., Chicago, IL, USA).

Lipid adjustment represents the relation of tocopherol to cholesterol + triacylglycerols in serum.

δ -Tocopherol was below detection limit.

TABLE A-6

Concentration of serum lipids and uric acid during the 5-day standardised diet with 3-day urine collection following consumption of black currant seed press residue in bread (means \pm SD; $n = 36$).

Parameters	Unit	Baseline	Control bread	Test bread
Total cholesterol	[mmol/l]	4.91 \pm 1.07	5.04 \pm 1.11	4.90 \pm 1.22
HDL cholesterol	[mmol/l]	1.64 \pm 0.47	1.68 \pm 0.55	1.64 \pm 0.52
LDL cholesterol	[mmol/l]	3.03 \pm 0.96	3.02 \pm 0.90	2.92 \pm 0.95
LDL/HDL cholesterol		1.96 \pm 0.75	1.94 \pm 0.76	1.91 \pm 0.78
Triacylglycerols	[mmol/l]	0.98 \pm 0.46 ^b	1.10 \pm 0.52 ^a	1.02 \pm 0.58 ^{a,b}
Uric acid	[μ mol/l]	214 \pm 46	204 \pm 49	214 \pm 54

^{a,b} Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

Parameters were achieved using the Abbott Architect® c8000 analyzer and the corresponding test kits (Abbott, Wiesbaden, Germany).

Statistical evaluation was carried out using analysis of variance for repeated measures (SPSS 14.0 software package, SPSS Inc., Chicago, IL, USA).

TABLE A-7

Concentration of the faecal short-chain fatty acids and neutral sterols during the 5-day standardised diet with 3-day stool collection following consumption of black currant seed press residue in bread (means \pm SD; $n = 36$).

Parameters	Baseline	Control bread	Test bread
[$\mu\text{mol/g}$]			
Total short-chain fatty acids	80.3 \pm 43.6	77.1 \pm 26.5	82.2 \pm 22.3
Acetate	47.1 \pm 24.3	46.0 \pm 15.8	49.0 \pm 13.7
Propionate	15.0 \pm 9.8	12.8 \pm 5.4	12.8 \pm 3.5
<i>i</i> -Butyrate	1.53 \pm 0.55	1.63 \pm 0.63	1.64 \pm 0.34
<i>n</i> -Butyrate [†]	12.1 \pm 10.8	11.6 \pm 5.6	13.7 \pm 6.9
<i>i</i> -Valerate	2.11 \pm 0.92 ^b	2.61 \pm 1.23 ^a	2.65 \pm 0.59 ^a
<i>n</i> -Valerate	1.80 \pm 0.78	1.88 \pm 0.74	1.85 \pm 0.54
<i>n</i> -Capronate [†]	0.65 \pm 1.22	0.60 \pm 0.82	0.50 \pm 0.55
[mg/g fresh matter]			
Coprostanol	4.19 \pm 3.36	3.78 \pm 2.84	3.50 \pm 2.54
Cholesterol [†]	1.59 \pm 1.43 ^a	1.08 \pm 1.26 ^b	1.08 \pm 1.37 ^b
Coprostanon [†]	0.417 \pm 0.560	0.364 \pm 0.448	0.337 \pm 0.406
Cholestanol	0.140 \pm 0.052 ^a	0.114 \pm 0.046 ^b	0.113 \pm 0.038 ^b
Cholestanon [†]	0.003 \pm 0.005	0.003 \pm 0.002	0.003 \pm 0.003
Total neutral sterols	6.34 \pm 3.20 ^a	5.34 \pm 2.52 ^b	5.03 \pm 2.32 ^b

^{a,b} Mean values within a row with dissimilar superscript letters were significantly different ($P \leq 0.05$). Results without superscripts in a row had no significant differences.

Statistical evaluation was carried out using analysis of variance for repeated measures (SPSS 14.0 software package, SPSS Inc., Chicago, IL, USA).

[†] Values were not distributed normally; significance was calculated by means of the Wilcoxon test.

SELBSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Die vorliegende Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt.

Jena, 22.06.2009

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Dorit Helbig

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Jena, 22.06.2009

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Dorit Helbig

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LIST OF PUBLICATIONS, ORAL PRESENTATIONS AND POSTERS

PUBLICATIONS

Nanoscale and customary non-esterified sitosterols are equally enriched in different body compartments of the guinea pig

Sylvia Keller, Dorit Helbig, Albert Härtl, Gerhard Jahreis

Molecular Nutrition & Food Research 51:1503-1509 (2007)

Berry seed press residues and their valuable ingredients with special regard to black currant seed press residues

Dorit Helbig, Volker Böhm, Andreas Wagner, Rainer Schubert, Gerhard Jahreis

Food Chemistry 111:1043-1049 (2008)

Black currant seed press residue increases tocopherol concentrations in serum and stool whilst biomarkers in stool and urine indicate increased oxidative stress in humans

Dorit Helbig, Andreas Wagner, Michael Glei, Samar Basu, Rainer Schubert,
Gerhard Jahreis

British Journal of Nutrition Accepted 5 December 2008

(doi:10.1017/S0007114509220812)

Tocopherol isomer pattern in serum and stool of human following consumption of black currant seed press residue administered in whole grain bread

Dorit Helbig, Andreas Wagner, Rainer Schubert, Gerhard Jahreis

Clinical Nutrition Under review (submitted 27 November 2008)

ORAL PRESENTATIONS AND POSTERS

Einfluss einer Phytosterolsupplementation auf die Sterolverteilung in spezifischen Körpergeweben am Meerschweinchen-Modell

Dorit Ulbricht, Sylvia Keller, Gerhard Jahreis

Proceedings of the German Nutrition Society, Abstracts zum 42. Wissenschaftlichen Kongress, Vol. 7:73 (2005)

Akkumulation von unverestertem Sitosterol verschiedener Partikelgrößen in Geweben beim Meerschweinchen

Dorit Ulbricht, Sylvia Keller, Gerhard Jahreis

13th Atherosklerose-Symposium, Erfurt, Germany (June 17-18, 2005)

Accumulation of non-esterified sitosterol different in particle size in the tissues of the guinea pig

Dorit Ulbricht, Sylvia Keller, Gerhard Jahreis

10th Symposium "Vitamins and Additives in the nutrition of men and animals", Jena, Germany (September 28-29, 2005)

Fat-soluble constituents of physiological importance in differently processed berry seeds or seed fractions with special regard to tocopherols and tocotrienols

Dorit Ulbricht, Andreas Wagner, Sylvia Keller, Volker Böhm., Rainer Schubert

13th European meeting on fat-soluble vitamins, Dornburg, Germany (March 30-April 1, 2006)

Antioxidative capacity in human faeces after intervention with black currant seed press residues

Dorit Ulbricht, Andreas Wagner, Sandra Schulze, Gerhard Jahreis, Rainer Schubert

10th Karlsruhe Nutrition Congress, "Health aspects of vegetables and fruits: Scientific evidence for 5-a-day", Karlsruhe, Germany (October 15-17, 2006)

Intervention with berry seed press residues – effects on markers regarding antioxidant status

Dorit Helbig, Andreas Wagner, Claudia Nitsch, Rainer Schubert, Gerhard Jahreis

First international symposium on secondary metabolism in plant seeds: Current status and future applications, Potsdam, Germany (February 21-24, 2007)

Schwarze Johannisbeersamen, reich an Tocopherolen - Physiologische Effekte nach Verzehr

Dorit Helbig, Andreas Wagner, Claudia Nitsch, Gerhard Jahreis

42. Vortragstagung der Deutschen Gesellschaft für Qualitätsforschung, "Antioxidantien in pflanzlichen Lebensmitteln", Kiel, Germany (March 19-20, 2007)

Einfluss einer Intervention mit Johannisbeersamen-Pressrückständen auf die Serum- und Stuhl-Tocopherolkonzentration sowie Marker des oxidativen Stresses

Dorit Helbig, Andreas Wagner, Rainer Schubert, Gerhard Jahreis

Proceedings of the German Nutrition Society, Abstracts zum 45. Wissenschaftlichen Kongress, Vol. 12:63 (2008)